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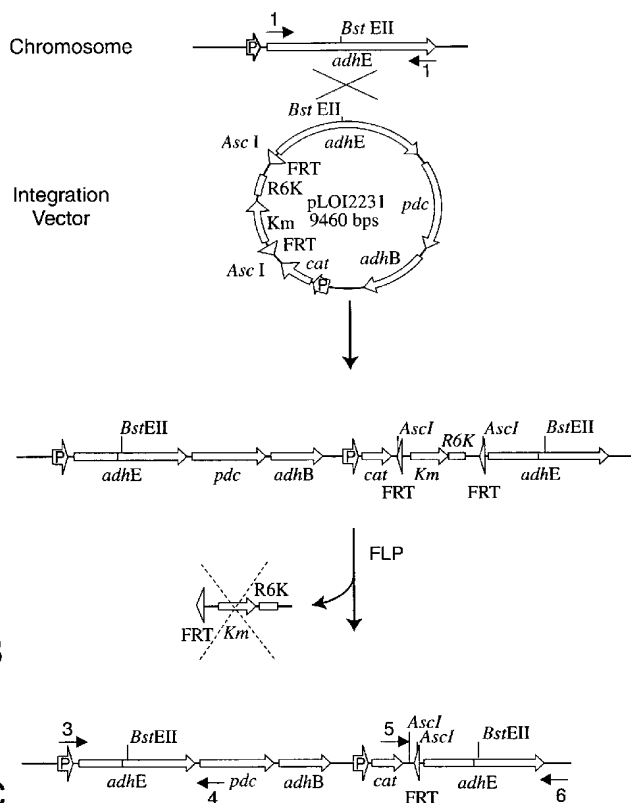
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(54) Title: METHODS AND COMPOSITIONS FOR THE CHROMOSOMAL INTEGRATION OF HETEROLOGOUS SEQUENCES



(57) Abstract: The invention provides gene constructs with *in vivo* excisable selection markers, and methods for their use, that are suitable for use in the chromosomal integration of heterologous DNA. Ethanologenic host cells, and methods for engineering such cells, are also provided.



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METHODS AND COMPOSITIONS FOR THE CHROMOSOMAL INTEGRATION OF HETEROLOGOUS SEQUENCES

**Related Information**

- 5           The contents of the patents, patent applications, and references cited throughout this specification are hereby incorporated by reference in their entireties.

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**Background of the Invention**

- 15           Plasmid vectors are versatile tools which facilitate the isolation, expression and analysis of genes (Bolivar, F., *et al.*, *Gene* 2: 95-113 (1977)). Useful characteristics include the facile production of identical DNA for subsequent *in vitro* and *in vivo* manipulation, the presence of multiple cloning sites (MCS), selectable markers which allow rapid screening for new or improved traits, and the ease with which they can be  
20           established as multiple cellular copies to alter gene expression in recombinant hosts. However, the physiological burden imposed by multiples copies of plasmid genes, potential for internal re-arrangements, and segregational instability are disadvantages for many biotechnological applications (Peredelchuk, M. Y., *Gene* 187: 231-238 (1997)).

          Antibiotic-resistance genes are frequently used for plasmid maintenance.

- 25           Alternative selectable markers based on metabolic deficiencies of the host (Degryse, E., *J. Biotech.* 18: 29-40 (1991)) pose further complications for improvement cycles in production strains. For applications such as the deliberate field release, development of organisms for use in food products, and development of biocatalysts for bulk chemicals, special requirements for plasmid maintenance are undesirable.

- 30           Many of the problems associated with plasmids can be eliminated by the chromosomal integration of desired traits. Integration tools based on modified transposons and conditional plasmid replicons have been developed (de Lorenzo, V., *et*

*al.*, *J. Bacteriol.* 172: 6568-6572 (1990); de Lorenzo, V., *et al.*, *Methods Enzymol.* 235: 386-405 (1994); Rode, C. K., *et al.*, *Gene*. 166: 1-9 (1995); Hamilton, C. H., *et al.*, *J. Bacteriol.* 171: 4617-4622 (1989); Kaniga, K., *et al.*, *J. Bacteriol.* 109: 137-141 (1991); Le Borgne, S., *et al.*, *Gene* 223: 213-219 (1998); Link, A.J., *et al.*, *J. Bacteriol.* 179: 6228-6237 (1997)). With these tools, integration can be random or precisely directed by DNA fragments homologous to the host genome. However, complications still remain with most integration systems such as the persistence of selectable markers, transposons, or replicons. For strains in which multiple alterations or continuing improvements are desired, the accumulation of markers and delivery systems can be troublesome.

Selectable events may be limited by the availability of functional markers. Integrated DNA (replicons, transposon genes, and selectable marker genes) can serve as a site for homologous recombination events which interfere with targeting or randomness during subsequent constructions. Also, the persistence of replicons and transposons increase the potential for gene transfer to other organisms in the environment.

Replicons and transposons can be eliminated by transforming with purified DNA fragments which lack replication functions ( Hasan, N., *et al.*, *Gene* 150: 51-56 (1994); Ohta, K., *et al.*, *Appl. Environ. Microbiol.* 57: 893-900 (1991)). Non-antibiotic markers are available but are often less efficient than antibiotics (de Lorenzo, V., *et al.*, *J. Bacteriol.* 172: 6568-6572 (1990); de Lorenzo, V., *et al.*, *Methods Enzymol.* 235: 386-405 (1994); Herrero, M., *et al.*, *J. Bacteriol.* 172: 6557-6567 (1990)). In a few cases, loss of functions such as tetracycline-sensitivity and absence of sucrose-*sacB* system can be selected directly (Bochner, B. R., *et al.* *J. Bacteriol.* 143: 926-933 (1980); Kaniga, K., *et al.*, *J. Bacteriol.* 109: 137-141 (1991); Ried, J.L., *et al.*, *Gene* 57: 239-246 (1985)). However, loss of function due to a mutation is typically not a precise event and can result from unstable point mutations, partial deletion of the resistance gene, or extended deletions which impair the host.

### **Summary of the Invention**

The foregoing limitations are overcome using the method and vectors of the present invention. In particular, the invention provides a method for integrating nucleic acids into a genome in such a way that any unwanted vector or selectable marker DNA can be removed. This allows for the genome of the recipient host cell to be made

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substantially free of any unnecessary nucleic acid, *e.g.*, vector sequence, marker sequence, that can lead to genomic instability.

Accordingly, in one aspect, the invention provides a method for integrating a nucleic acid construct into the genome of a host cell by contacting the cell with a nucleic acid construct under conditions such that the nucleic acid construct is integrated by the cell. The method uses a nucleic acid construct that contains a passenger sequence and a marker sequence, where the marker sequence is flanked by a first and second recombining site. In one embodiment, the first and second recombining site are oriented in the same direction.

10 In another embodiment, the method employs a construct that further contains an origin of replication between the first and second recombining sites, *e.g.*, a conditional origin of replication (*i.e.*, replicon) preferably, *e.g.*, pSC101ori, R6K- $\gamma$ ori, colE1, oriEV, or an origin of replication derived therefrom.

In another embodiment, the nucleic acid construct of the above aspect contains a sequence that contains a promoter, a restriction site, an intron, an exon, an IRES element, a polyadenylation site, or a combination thereof.

In yet another related embodiment, the nucleic acid construct of the above aspect contains a guide sequence capable of directing site-specific integration of the nucleic acid construct to a specific site in the sequence of a replicating genome.

20 In even another embodiment, the above method involves exposing the targeted cell to a site-specific recombinase that results in recombination between the first and second recombining sites of the foregoing nucleic acid construct such that the intervening sequence flanked by the recombining sites is excised. The method involves using a recombinase such as Xer, Int, Cre, and preferably, FLP recombinase.

25 Accordingly, in a related embodiment, the corresponding recombining sites are dif, att, loxP, and preferably FRT. In a preferred embodiment, the above method can be repeated such that the sequential introduction of more than one genetic element may be introduced.

In still another embodiment, the passenger sequence encodes at least one gene, preferably a gene involved in ethanologenesis, such as, for example, *adh* or *pdc*. In a related embodiment, the gene involved in ethanologenesis may be derived from a

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prokaryote or a eukaryote. In another related embodiment, the passenger sequence may further contain a promoter 5' to the passenger sequence.

In another embodiment, the above method employs a nucleic acid construct containing a marker sequence that encodes a selectable gene, *e.g.*, an antibiotic  
5 resistance gene or a non-antibiotic resistance gene. In a preferred embodiment, the antibiotic resistance gene is the gentamycin resistance gene, zeocin resistance gene, kanamycin resistance gene, ampicillin resistance gene, tetracycline resistance gene, or chloramphenicol resistance gene. In another preferred embodiment, the non-antibiotic  
10 resistance gene is an auxotrophic gene, a metal ion resistance gene, a *trp* gene, or a gene encoding a green fluorescent protein.

In a preferred embodiment of the first aspect, the method employs a bacterial cell, preferably a Gram-negative bacterial cell, more preferably a facultatively anaerobic bacterial cell, more preferably a bacterial cell selected from the family  
Enterobacteriaceae, and most preferably, a bacterial cell of the genus *Klebsiella* or  
15 *Escherichia* (*e.g.*, *E. coli* B or *E. coli* K12). In a related embodiment, the host cell is a recombinant bacterial cell. In another related embodiment, the method uses a nucleic acid construct that is, or is derived from, a plasmid selected from the group consisting of pLOI2223, pLOI2224, pLOI2225, pLOI2226, pLOI2227, pLOI2228, and pLOI2403.

In a second aspect, the invention provides a method for producing a recombinant  
20 ethanologenic cell by contacting a cell with a nucleic acid construct under conditions in which integration of the nucleic acid construct occurs resulting in the formation of a recombinant ethanologenic cell. The method uses a nucleic acid construct that contains a passenger sequence that contains an ethanologenic gene, and a marker sequence, flanked by a first and second recombining site. In one embodiment, the first and second  
25 recombining site are oriented in the same direction.

In another embodiment, the passenger sequence encodes an ethanologenic gene such as *adh* or *pdc*. In a related embodiment, the passenger sequence encodes *pdc*, *adhB*, and *cat*.

In another embodiment, the nucleic acid further contains a guide sequence  
30 thereby resulting in site-specific integration of the nucleic acid construct. In a related embodiment, the guide sequence is derived from a replicating genome.

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In yet another embodiment, the method employs a construct that further contains an origin of replication between the first and second recombining sites, *e.g.*, a conditional origin of replication (*i.e.*, replicon) preferably, *e.g.*, pSC101ori, R6K- $\gamma$ ori, colE1, oriEV, or an origin of replication derived therefrom.

- 5           In even another embodiment, the above method involves exposing the targeted cell with a site-specific recombinase that results in recombination between the first and second recombining sites of the foregoing nucleic acid construct such that the intervening sequence flanked by the recombining sites is excised. The method involves using a recombinase such as Xer, Int, Cre, and preferably, FLP recombinase.
- 10         Accordingly, in a related embodiment, the corresponding recombining sites are dif, att, loxP, and preferably FRT.

- In still another embodiment, the above method employs a nucleic acid construct containing a marker sequence that encodes a selectable gene, *e.g.*, an antibiotic resistance gene or a non-antibiotic resistance gene. In a preferred embodiment, the
- 15          antibiotic resistance gene is the gentamycin resistance gene, zeocin resistance gene, kanamycin resistance gene, ampicillin resistance gene, tetracycline resistance gene, or chloramphenicol resistance gene. In another preferred embodiment, the non-antibiotic resistance gene is an auxotrophic gene, a metal ion resistance gene, a *trp* gene, or a gene encoding a green fluorescent protein.

- 20           In a preferred embodiment of the foregoing aspect, a recombinant ethanologenic cell is produced according to the above method.

- In a third aspect, the invention provides a recombinant host cell having a nucleic acid construct that contains a passenger sequence and a marker sequence, flanked by a first and second recombining site. In one embodiment, the first and second recombining
- 25          site are oriented in the same direction.

            In a related embodiment, the host cell contains a nucleic acid construct where the passenger sequence encodes a gene involved in ethanogenesis such as, *e.g.*, *adh* or *pdc*. In a related embodiment, the passenger sequence is selected from the group including *adh*, *pdc*, and *cat*.

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In one embodiment, the host cell contains a nucleic acid construct that further contains an origin of replication between the first and second recombining sites, *e.g.*, a conditional origin of replication (*i.e.*, replicon) preferably, *e.g.*, pSC101ori, R6K- $\gamma$ ori, colE1, oriEV, or a an origin of replication derived therefrom.

5 In another embodiment, the host cell contains a nucleic acid construct containing a guide sequence capable of directing site-specific integration of the nucleic acid construct to a specific site in the sequence of a replicating genome.

In yet another embodiment, the host cell contains a nucleic acid construct containing a marker sequence that encodes a selectable gene, *e.g.*, an antibiotic  
10 resistance gene or a non-antibiotic resistance gene. In a preferred embodiment, the antibiotic resistance gene is the gentamycin resistance gene, zeocin resistance gene, kanamycin resistance gene, ampicillin resistance gene, tetracycline resistance gene, or chloramphenicol resistance gene. In another preferred embodiment, the non-antibiotic resistance gene is an auxotrophic gene, a metal ion resistance gene, a *trp* gene, or a gene  
15 encoding a green fluorescent protein.

In another embodiment, the host cell is exposed to a site-specific recombinase that results in recombination between the first and second recombining sites of the foregoing nucleic acid construct such that the intervening sequence flanked by the recombining sites, is excised. In a preferred embodiment, the recombination involves  
20 using a recombinase such as Xer, Int, Cre, and preferably, FLP recombinase. Accordingly, in a related embodiment, the corresponding recombining sites are dif, att, loxP, and preferably FRT.

In a preferred embodiment, the host cell is a bacterial cell, *e.g.*, a recombinant cell and/or an ethanologenic cell, preferably a Gram-negative bacterial cell, more  
25 preferably a facultatively anaerobic bacterial cell, more preferably selected from the family Enterobacteriaceae, and most preferably, of the genus *Klebsiella* or *Escherichia* (*e.g.*, *E. coli* B or *E. coli* K12). In a related embodiment, the host cell contains, a nucleic acid construct that is, or is derived from, a plasmid selected from the group consisting of pLOI2223, pLOI2224, pLOI2225, pLOI2226, pLOI2227, pLOI2228, and pLOI2403.

30 In a fourth aspect, the invention provides a method for producing ethanol by providing a recombinant ethanologenic cell with a nucleic acid construct that contains a passenger sequence and a marker sequence, where the marker sequence is flanked by a



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first and second recombining site. The method includes contacting the cell with a substrate which can be fermented into ethanol, such that expression of the passenger sequence results in the production of ethanol.

5 In one embodiment, the first and second recombining site are oriented in the same direction.

In another embodiment, the passenger sequence encodes a gene involved in ethanologenesis, such as, *e.g.*, *adh* or *pdh*. In a related embodiment, the passenger sequence encodes *adhB*, *pdh*, and *cat*.

10 In another embodiment, the method employs a construct that further contains an origin of replication between the first and second recombining sites, *e.g.*, a conditional origin of replication (*i.e.*, replicon) preferably, *e.g.*, pSC101ori, R6K- $\gamma$ ori, colE1, oriEV, or a an origin of replication derived therefrom.

15 In yet another embodiment, the nucleic acid construct of the above aspect contains a guide sequence capable of directing site-specific integration of the nucleic acid construct to a specific site in the sequence of a replicating genome.

20 In yet another embodiment, the above method involves exposing the targeted cell with a site-specific recombinase that results in recombination between the first and second recombining sites of the foregoing nucleic acid construct such that the intervening sequence flanked by the recombining sites is excised. The method involves using a recombinase such as Xer, Int, Cre, and preferably, FLP recombinase. Accordingly, in a related embodiment, the corresponding recombining sites are dif, att, loxP, and preferably FRT.

25 In still another embodiment, the above method employs a nucleic acid construct containing a marker sequence that encodes a selectable gene, *e.g.*, an antibiotic resistance gene or a non-antibiotic resistance gene. In a preferred embodiment, the antibiotic resistance gene is the gentamycin resistance gene, zeocin resistance gene, kanamycin resistance gene, ampicillin resistance gene, tetracycline resistance gene, or chloramphenicol resistance gene. In another preferred embodiment, the non-antibiotic resistance gene is an auxotrophic gene, a metal ion resistance gene, a *trp* gene, or a gene  
30 encoding a green fluorescent protein.

In a preferred embodiment, the method employs a host cell that a bacterial cell, *e.g.*, a recombinant bacterial cell, preferably a Gram-negative bacterial cell, more preferably a facultatively anaerobic bacterial cell, more preferably selected from the family Enterobacteriaceae, and most preferably, either *Klebsiella* or *Escherichia* (*e.g.*, *E. coli* B or *E. coli* K12).

In one embodiment, the method uses a nucleic acid construct that is, or is derived from, a plasmid selected from the group consisting of pLOI2223, pLOI2224, pLOI2225, pLOI2226, pLOI2227, pLOI2228, and pLOI2403.

In a fifth aspect, the invention provides a nucleic acid construct containing a passenger sequence, and a marker sequence, where the marker sequence is flanked by a first and second recombining site. In a related embodiment the first and second recombining site may be oriented in the same direction.

In another embodiment, the construct further contains an origin of replication between the first and second recombining sites, *e.g.*, a conditional origin of replication (*i.e.*, replicon) preferably, *e.g.*, pSC101ori, R6K- $\gamma$ ori, colE1, oriEV, or a an origin of replication derived therefrom.

In another embodiment, the nucleic acid construct contains a guide sequence derived from a replicating genome. In a related embodiment, the guide sequence is derived from a bacterial cell.

In yet another embodiment, the construct further contains at least one unique restriction enzyme site. In yet another related embodiment, the passenger sequence of the construct contains an ethanologenic gene, such as, *e.g.*, *adh* or *pdh*, and preferably *adhB*, *pdh*, *cat* or a combination thereof.

In still another related embodiment, the passenger sequence of the construct contains a sequence selected from the group consisting of a heterologous promoter and a prokaryotic termination sequence.

In even another embodiment, the nucleic acid construct contains a marker sequence that encodes a selectable gene, *e.g.*, an antibiotic resistance gene or a non-antibiotic resistance gene. In a preferred embodiment, the antibiotic resistance gene is the gentamycin resistance gene, zeocin resistance gene, kanamycin resistance gene, ampicillin resistance gene, tetracycline resistance gene, or chloramphenicol resistance gene. In another preferred embodiment, the non-antibiotic resistance gene is an

auxotrophic gene, a metal ion resistance gene, a *trp* gene, or a gene encoding a green fluorescent protein.

In another embodiment, the construct is exposed to a site-specific recombinase that results in recombination between the first and second recombining sites of the  
5 nucleic acid construct such that the intervening sequence flanked by the recombining sites is excised. The excision involves using a recombinase such as Xer, Int, Cre, and preferably, FLP recombinase. Accordingly, in a related embodiment, the construct contains recombining sites such as dif, att, loxP, and preferably FRT.

In a preferred embodiment of the above aspect, the construct is, or is derived  
10 from, a plasmid selected from the group consisting of pLOI2223, pLOI2224, pLOI2225, pLOI2226, pLOI2227, pLOI2228, and pLOI2403.

In a related embodiment, the invention provides a kit including at least one of the foregoing nucleic acid constructs and instructions for use.

15 Other features and advantages of the invention will be apparent from the following detailed description and claims.

### **Brief Description of the Drawings**

*Figure 1* shows a schematic of various integration vectors and helper plasmids  
20 (see text for details).

*Figure 2* shows a schematic illustrating the use of an integration vector for the insertion of heterologous “passenger” genes into a host genome. A “guide” sequence allows for the site specific targeting of the passenger sequence via homologous recombination. A helper plasmid provides a recombinase (FLP) that catalyzes the *in*  
25 *vivo* excision of any unnecessary sequence (*e.g.*, the replicon and marker sequence) flanked by recombining sites (FRT) leaving resident in the genome the passenger gene.

*Figure 3* shows a diagram illustrating the targeting of heterologous “passenger” genes to a site specific region of a genome (*i.e.*, the *adhE* gene (shaded)) and recombinase-mediated deletion of the replicon and marker used during plasmid  
30 construction and initial integration. The alignment of the guide sequence of the targeting vector and the genomic integration site (Panel A), cross-over event (Panel B),

and resultant recombinase-mediated (FLP) excision of the replicon and selectable marker (Panel C) are shown.

*Figure 4* is a photograph of an agarose gel stained with ethidium bromide showing PCR amplified nucleic acid fragments amplified using different primers (Panel A) and restriction enzyme digested (Panel B) to confirm the correct chromosomal  
5 A) and restriction enzyme digested (Panel B) to confirm the correct chromosomal integration of a heterologous nucleic acid (see text for details).

### **Detailed Description of the Invention**

In order for the full scope of the invention to be clearly understood, the following  
10 definitions are provided.

#### ***I. Definitions***

As used herein the term “host cell” and “recombinant host cell” is intended to include a cell suitable for genetic manipulation, *e.g.*, which can incorporate heterologous  
15 polynucleotide sequences, *e.g.*, which can be transfected. The cell can be a microorganism or a higher eukaryotic cell, such as an animal cell or a plant cell. The term is intended to include progeny of the cell originally transfected. In preferred embodiments, the cell is a bacterial cell, *e.g.*, a Gram-negative bacterial cell, and this term is intended to include all facultatively anaerobic Gram-negative cells of the family  
20 Enterobacteriaceae such as *Escherichia*, *Shigella*, *Citrobacter*, *Salmonella*, *Klebsiella*, *Enterobacter*, *Erwinia*, *Kluyvera*, *Serratia*, *Cedecea*, *Morganella*, *Hafnia*, *Edwardsiella*, *Providencia*, *Proteus*, and *Yersinia*. Particularly preferred recombinant hosts are *Escherichia coli* or *Klebsiella oxytoca* cells. Preferably, the term recombinant host cell is intended to include a cell that has already been selected or engineered to have certain  
25 desirable properties and suitable for further modification using the compositions and methods of the invention.

The term “passenger sequence” is intended to include any desired sequence that is intended for integration into the host cell. For example, the passenger sequence may include, a restriction site, multiple restriction sites (*e.g.*, a polylinker or multiple cloning  
30 site (MCS)), a unique stretch of sequence suitable for marking the cell as distinct from a wild type cell, a regulatory element, *e.g.*, a polyadenylation sequence, a promoter, an intron, a splice signal, an internal ribosomal entry site (IRES); for regulating the

expression of multiple genes or cistrons, and/or a gene, for example a gene encoding a polypeptide. A passenger sequence encoding a gene may further include a promoter if appropriate or use the endogenous promoter found proximal to the site of integration. In addition, the passenger sequence encoding a gene may further include other regulatory  
5 elements, if appropriate, to improve expression. A passenger sequence may comprise genetic elements derived from any source, *e.g.*, eukaryotes, prokaryotes, virii, or synthetic polynucleotide fragments.

The term "guide sequence" is intended to include a sequence that can be located 5', 3', or internal to the sequence intended to be integrated, such that recombination  
10 between the introduced vector (or portion thereof) and the recipient host cell genome is accomplished. Typically, a guide sequence has high similarity or identity with a site specific region of the recipient host cell genome such that a targeted integration of the passenger sequence to this site by homologous recombination is accomplished.

The term "site-specific integration" is meant to refer to the integration of an  
15 exogenous nucleic acid sequence to a specific area of the genome of a recipient host cell. In general, a guide sequence allows for the homologous recombination between a portion of the targeting vector and the host cell such that a predictable targeting of the sequence to that area of the genome represented in part by the guide sequence, is specifically targeted.

20 The term "marker sequence" is intended to include any sequence that can be encoded by a nucleic acid, introduced into the replicating genome of a recipient host cell, and detected, thereby indicating that the cell has been "marked" by such a sequence. Accordingly, the term is intended to include a sequence for example having a restriction enzyme sequence that can be detected by a corresponding restriction enzyme.  
25 In addition, or alternatively, the sequence may be detected using any of a variety of art recognized techniques, *e.g.*, polymerase chain reaction using appropriate primers, nucleic acid hybridization, *etc.* Preferably, the marker sequence encodes a gene product that confers on the cell a selectable phenotype, *e.g.*, resistance to an antibiotic or other cytotoxic agent or a conditional growth advantage. Accordingly, a marker sequence can  
30 encode, *e.g.*, the gentamycin resistance gene, zeocin resistance gene, kanamycin resistance gene, ampicillin resistance gene, tetracycline resistance gene, chloramphenicol resistance gene, hygromycin resistance gene, thymidine kinase, an

auxotrophic gene, a metal ion resistance gene, a *trp* gene, a gene producing a visual phenotype (*e.g.*, green fluorescent protein), or a gene producing a cell surface antigen (*e.g.*, CD4). Any of the foregoing markers when expressed in cells can be detected using art recognized techniques such as, *e.g.*, appropriate culture conditions or selection  
5 techniques (*e.g.*, antibodies, FACS (fluorescein activated cell sorting), or flow cytometric analysis).

The term “origin of replication” or “replicon” is intended to include any sequence, conditional or otherwise, that can confer on a nucleic acid sequence the ability to be replicated in a cell for the purposes of, *e.g.*, propagating the nucleic acid or  
10 maintaining the presence of the nucleic acid in a cell. Such sequences are known in the art and are routinely incorporated into the backbone of many nucleic acid vectors to facilitate their propagation and typically include, *e.g.*, pSC101ori, R6K- $\gamma$ ori, colE1ori, or oriEV.

The term “recombining site” is intended to include a nucleic acid element that  
15 represents a site specific binding site for a recombinase. Examples of such elements include, *e.g.*, the FRT sequence, the dif sequence, the att sequence, and the loxP sequence.

The term “recombinase” is intended to include any recombinase having a site specific recombinase activity. Examples of such recombinases are FLP, Xer, Int, and  
20 Cre and these enzymes typically have site specific recombinase activity on, respectively, the FRT sequence, the dif sequence, the att sequence and the loxP sequence.

The term “gene involved in ethanologenesis” is intended to include any gene capable of conferring on a cell ethanologenic properties or capable of improving any aspect of cellular ethanologenesis, such as, *e.g.*, substrate uptake, substrate processing,  
25 ethanol tolerance, *etc.* Genes involved in ethanologenesis are, *e.g.*, alcohol dehydrogenase, pyruvate decarboxylase, secretory protein/s, and polysaccharases, and these genes, or their homologs, may be derived from any appropriate organism.

The term “substrate” is intended to include any moiety that can be converted into ethanol. Substrates that are suitable for converting into ethanol include sugar moieties  
30 such as, *e.g.*, monosaccharides, disaccharides, trisaccharides, oligosaccharides, and complex carbohydrates, including, *e.g.*, lignocellulose, which comprises cellulose, hemicellulose, and pectin.

The term “derived from” is intended to include the isolation (in whole or in part) of a polynucleotide segment from an indicated source. The term is intended to include, for example, direct cloning, PCR amplification, or artificial synthesis from, or based on, a sequence associated with the indicated polynucleotide source.

5           The term “ethanogenic” is intended to include the ability of a microorganism to produce ethanol from a carbohydrate as a primary fermentation product. The term is intended to include naturally occurring ethanogenic organisms, ethanogenic organisms with naturally occurring or induced mutations, and ethanogenic organisms which have been genetically modified.

10           The term “Gram-negative bacteria” is intended to include the art recognized definition of this term. Typically, Gram-negative bacteria include, for example, the family Enterobacteriaceae which comprises, among others, the species *Escherichia* and *Klebsiella*.

15   **II.    Nucleic Acid Constructs**

The invention provides a number of novel nucleic acid constructs that are suitable for targeting a heterologous nucleic acid sequence to the genome or chromosome of a recipient host cell.

20           The heterologous sequence or passenger sequence of the targeting vector can be any desirable sequence that is useful when introduced into a cell. Accordingly, the sequence may be a regulatory element (*e.g.*, a promoter, intron, splicing signal, internal ribosome entry site, polyadenylation signal) or a gene encoding a gene product, *e.g.*, a polypeptide, or a combination thereof (*e.g.*, a gene encoding a polypeptide that is under the transcriptional control of a promoter). In addition, the heterologous sequence may  
25   be targeted to any replicating genome, *e.g.* a bacterial cell, a yeast, an insect cell, a plant cell, or an animal cell. It is well known in the art as to what regulatory elements, *e.g.*, promoters, *etc.*, are suitable for use in, *e.g.*, a bacterial cell versus a plant or animal cell.

          If site specific integration of the passenger sequence is desired (as opposed to random integration) a guide sequence must be incorporated into the nucleic acid vector.  
30   Typically the guide sequence contains a portion of the genomic locus to be targeted. Considered design of this region allows for the accurate placement of the passenger

sequence anywhere in the genome, preferably, *e.g.*, under the control of a desirable endogenous promoter.

To facilitate the construction of the targeting vector, an origin of replication, or replicon, and a marker sequence is included in the vector. Specifically exemplified in  
5 the present invention are the markers encoding antibiotic resistance genes to ampicillin, kanamycin, and chloramphenicol, however it will be appreciated that other selectable markers may be used as described above. In part, the selection of an appropriate marker will depend on the organism to be targeted, *e.g.*, prokaryotic versus eukaryotic. The characteristics of the foregoing markers and their efficacies in various organisms is well  
10 known in the art.

In addition to an appropriate marker, an origin of replication or replicon is also incorporated into the targeting construct to facilitate propagation of the vector during the construction and/or initial integration of the vector. Specifically exemplified are the pSC101 and R6K- $\gamma$  replicons however other replicons, *e.g.*, colE1, and oriEV as well as  
15 replicons suitable for use in eukaryotic cells are also encompassed by the invention.

Importantly, the constructs of the invention possess site specific recombining sites that flank all of the vector sequence that, after targeting of the passenger sequence to the genome, is no longer necessary or desired. Indeed, one advantage of the invention is that following targeting and integration of the passenger sequence to the genome of  
20 the host cell, the *in vivo* excision of any undesired sequence can be accomplished using a recombinase that binds the site specific recombining sites contained in the integrated vector.

Critical for allowing the correct excision of the undesired vector sequence is the placement and directionality of the recombining sites. The novel vectors of the  
25 invention possess a first and a second recombining site that flank all of the replicon and marker sequence and are oriented in the same direction. This allows for the complete excision of all the vector sequence leaving behind the desired targeted passenger sequence and a minimal remainder of the joined recombining sites (68 bps).

While the FRT recombining site and corresponding FLP recombinase is  
30 exemplified (Senecoff, J., *et al. P.N.A.S.* 82:7270-7274 (1985); Szybalski, W., *Gene* 135:279-290 (1993); Hoang, T.T., *et al., Gene* 212:77-86 (1998)), a number of other site specific recombining sites (*e.g.*, dif, att, loxP) and corresponding recombinases (*e.g.*,



Xer, Int, Cre) may be used. Accordingly, any of the above described markers may be used after the excision step because the recipient host cell has once again been rendered sensitive to the selectable marker. Thus, one can sequentially introduce multiple genes into an organism without ever running into the difficulty of having to change markers or exhaust all suitable markers. Accordingly, if a particular selectable marker works efficiently in a particular organism, it may be exclusively used to introduce multiple passenger sequences into a target organism.

In a preferred embodiment, the constructs of the invention are modified to contain a passenger sequence encoding ethanol pathway gene products such as, *e.g.*, alcohol dehydrogenase (*e.g.*, *adhB*) and pyruvate decarboxylase (*pdc*), that are necessary for ethanol production in, *e.g.*, a microorganism. A guide sequence specific for the endogenous gene in the target organism allows for the targeting of these genes to a specific locus such that the heterologous ethanol pathway genes (*i.e.*, *adhB* and *pdc*) are brought under the transcriptional control of an appropriate endogenous promoter (*e.g.*, *adhE*).

Accordingly, the foregoing novel constructs allow for the genetic engineering of superior host cells suitable, *e.g.*, for various industrial applications, such as, *e.g.*, use in a fermentation reaction for producing, *e.g.*, ethanol (for a review of other industrial applications, see, *e.g.*, Barrios-Gonzalez *et al.*, *Biotechnol. Ann. Rev.* 2:85-121 (1996); *From Ethnomycology to Fungal Biotechnology: Exploiting from Natural Resources for Novel Products*, Singh, J., Ed., Plenum Press, Pub. (1999); *Manual of Industrial Microbiology and Biotechnology*, Demain, A. Ed., Am. Soc. of Microbiology, Pub., (1999); *Biomining: Theory, Microbes, and Industrial Processes*, Rawlings, Ed., R.G. Landes Co., Pub. (1997); *Biotechnology of Industrial Antibiotics*, Vandamme, E., Ed., Marcel Dekker, Pub. (1984); *Industrial Biotechnology*, Malik, V., Ed., Science, Pub. (1992); *Biotechnology and Food Ingredients*, Goldberg *et al.*, Ed., Aspen Publishers (1991); *Biotechnology and Food Process Engineering*, Schwartzberg *et al.*, Ed., Marcel Dekker, Pub. (1990); and *Food Biotechnology: Techniques and Applications*, Mittal, G., Technomic Pub. Co. (1992).

### III. Methods of Use

In a preferred embodiment, the methods of the invention apply the constructs described above for the integration of one or more desirable gene sequences into a host cell. Accordingly, the methods of the invention have immediate application in the genetic engineering of, *e.g.*, microorganisms (*e.g.*, for various industrial applications), plants (*e.g.*, for pest resistance, hardiness, yields), and animal cells (*e.g.*, for producing cytokines, hormones, *etc.*).

In one particular application of the methods of the invention, one or more genes necessary for producing ethanol are provided on a plasmid or integrated into a host chromosome using the method of the invention. More preferably, essential genes for fermenting a sugar substrate into ethanol, *e.g.*, pyruvate decarboxylase (*e.g.*, *pdh*) and/or alcohol dehydrogenase (*e.g.*, *adh*, preferably *adhB*) are introduced into the host of the invention using a bicistronic operon or PET operon as described in U.S.P.N. 5,821,093, hereby incorporated by reference.

Art recognized techniques for introducing nucleic acids into prokaryotic or eukaryotic cells such as *e.g.*, bacteria, yeast, insects, plants, and animals as well as appropriate culturing methods have been described (see, *e.g.*, *Large-Scale Mammalian Cell Culture Technology*, Lubiniecki, A., Ed., Marcel Dekker, Pub., (1990), *Bacterial Cell Culture: Essential Data*, Ball, A., John Wiley & Sons, (1997), *Molecular and Cell Biology of Yeasts*, Yarranton *et al.*, Ed., Van Nostrand Reinhold, Pub., (1989); *Yeast Physiology and Biotechnology*, Walker, G., John Wiley & Sons, Pub., (1998); *Baculovirus Expression Protocols*, Richardson, C., Ed., Humana Press, Pub., (1998); *Methods in Plant Molecular Biology: A Laboratory Course Manual*, Maliga, P., Ed., C.S.H.L. Press, Pub., (1995); *Current Protocols in Molecular Biology*, eds. Ausubel *et al.*, John Wiley & Sons (1992), Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual. 2nd, ed.*, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), and *Bergey's Manual of Determinative Bacteriology*, Kreig *et al.*, Williams and Wilkins (1984)).

Accordingly, using the methods of the invention, a single genetic construct can be designed to encode all of the necessary gene products (*e.g.*, a glucanase, an endoglucanase, an exoglucanase, a secretory protein/s, pyruvate decarboxylase, alcohol

dehydrogenase) for imparting to a non-ethanologenic (or weakly ethanologenic organism) all the necessary genes for producing ethanol from a sugar substrate.

Alternatively, the invention allows for more than one gene to be introduced serially without having to change markers. In a preferred embodiment, this allows for  
5 the engineering of a host cell suitable for performing simultaneous saccharification and fermentation (SSF). In addition, it will also be appreciated that such a host may be further manipulated, using methods known in the art, to have mutations in any endogenous gene/s (*e.g.*, recombinase genes) that would interfere with the stability, expression, and function of the introduced genes. Further, it will also be appreciated that  
10 the invention is intended to encompass any regulatory elements, gene/s, or gene products, *i.e.*, polypeptides, that are sufficiently homologous to the ones described herein.

Methods for screening strains having the introduced genes may be performed using, *e.g.*, PCR amplification using site or gene specific primers, visual screens that can  
15 identify cells expressing either the desired integrated gene, marker, or absence of the marker and any other unnecessary nucleic acid or other art recognized techniques.

For example, for screening an ethanologenic microorganism, the ADH gene product produces acetaldehyde that reacts with the leucosulfonic acid derivative of p-roseaniline to produce an intensely red product. Thus, ADH-positive clones can be  
20 easily screened and identified as bleeding red colonies.

Rapid evaluations of ethanol producing potential can also be made by testing the speed of red spot development on aldehyde indicator plates (Conway *et al.*, (1987) *J. Bacteriol.* 169:2591-2597). Typically, strains which prove to be efficient in sugar conversion to ethanol can be recognized by the production of red spots on aldehyde  
25 indicator plates within minutes of transfer.

In a most preferred embodiment of the invention, the methods of the invention allow for the production of a single host cell that is ethanologenic, that is, has all the necessary genes, either naturally occurring or artificially introduced or enhanced (*e.g.*, using a surrogate promoter and/or genes from a different species or strain), such that the  
30 host cell has the ability to produce and secrete a polysaccharase/s, degrade a complex sugar, and ferment the degraded sugar into ethanol. Accordingly, such a host is suitable for simultaneous saccharification and fermentation. Moreover, this host cell is free of

marker sequences, unnecessary vector sequence, a heterologous replicon, and these sequences can lead to genomic instability in the organism or make the organism less suitable for release or use outside, *e.g.*, a controlled laboratory environment.

In addition, it should be readily apparent to one skilled in the art that the ability  
5 conferred by the present invention, to transform genes coding for a protein or an entire metabolic pathway into a host cell without leaving resident in the host genome unnecessary or undesired nucleic acid has wide application. Envisioned in this regard, for example, is the application of the present invention to a variety of situations where regulatory elements and/or genes may be added at will and moreover, deleterious genes  
10 deleted, without unnecessarily littering the genome of the target organism with unwanted nucleic acid.

#### ***IV. Host Cells***

The invention also relates to host cells that are modified using the foregoing  
15 methods and/or nucleic acid constructs of the invention. Preferably, any host cell, *e.g.*, animal, plant, or microbe, can be modified according to the methods of the invention. More preferably, the methods and constructs of the invention allow for the engineering of heterologous DNA into the genome of any microorganism suitable, *e.g.*, for an industrial application. Even more preferably, the invention provides for the engineering  
20 of an organism useful for the production of ethanol, *e.g.*, by fermentation.

Accordingly, in one embodiment, a host cell of the invention comprises a heterologous polynucleotide segment encoding a polypeptide under the transcriptional control of a naturally occurring promoter in the genome or, if appropriate, under the control of a heterologous promoter.

25 In another embodiment, the host cell prepared according to the method of the invention is exposed to a recombinase such that the *in vivo* excision of any unnecessary nucleic acid flanked by recombining sites is accomplished while the targeted passenger sequence encoding a desirable gene is left behind. Typically, the nucleic acid sequence between the recombining sites contains, for example, a marker sequence and may further  
30 contain an origin of replication or replicon. The host cell of the invention is render free of this sequence by being exposed to a site specific recombinase, preferably encoded by an expressible construct, which is introduced into the cell using standard techniques.

In a preferred embodiment, the resultant recombinant host cell is made substantially free of any nucleic acid flanked by FRT recombining sites using an FLP recombinase. It is understood that the host cell of the invention may also be modified using nucleic acid constructs having recombining sites such as a dif sequence, att  
5 sequence, or loxP sequence and made substantially free of any undesired nucleic acid using a corresponding recombinase, such as, *e.g.*, Xer, Int, or Cre.

The resultant host cell as prepared according to the method of the invention has the advantage of no longer having any unnecessary heterologous DNA which can lead to, *e.g.*, genomic stability. In addition, the host cell no longer encodes a marker  
10 sequence and thus can be retargeted with the same selectable marker sequence in order to introduce another genetic modification to the cell. Thus, the serial introduction of multiple genes can be achieved using the same selection marker. In theory at least, there appears to be no limit as to the number of gene constructs that can be introduced into a host cell of the invention.

15 In a most preferred embodiment, the host cell of the invention contains at least one passenger sequence containing, *e.g.*, a gene of interest, *e.g.*, a gene encoding a desired polypeptide for use in the bioconversion of a sugar to ethanol, or a step thereof. Preferred ethanologenic genes include those that encode, *e.g.*, any ethanol pathway gene that can facilitate the production of ethanol from the cell or extract thereof, such as  
20 alcohol dehydrogenase, pyruvate decarboxylase, a cellulase, or a secretory polypeptide. A desired ethanologenic gene may be derived from another species, *e.g.*, a yeast, an insect, an animal, or a plant. The techniques for introducing and expressing such genes in a recombinant host, are presented in the examples.

For example, a non-ethanologenic host cell may be converted into an  
25 ethanologenic host by introducing, for example, ethanologenic genes from an efficient ethanol producer like *Zymomonas mobilis*. This type of genetic engineering, using the constructs and methods of the invention, results in a recombinant host capable of efficiently fermenting sugar into ethanol.

Accordingly, the invention makes use of a non-ethanologenic recombinant host,  
30 *e.g.*, *E. coli* strain B or *E. coli* strain K-12. These strains may be used to express a desired polypeptide, *e.g.*, an ethanologenic gene introduced using techniques describe herein. In a preferred embodiment of the present invention, the host cell having been

- 20 -

modified using the methods and constructs of the invention, can be applied in degrading or depolymerizing a complex saccharide into monosaccharides. Subsequently, the host cell can catabolize the simpler sugar into ethanol by fermentation. This process of concurrent complex saccharide depolymerization into smaller sugar residues followed  
5 by fermentation is referred to as simultaneous saccharification and fermentation (SSF).

In another embodiment, the invention makes use of a recombinant host that is ethanologenic and is improved using the methods of the invention. It is understood that an improvement of an ethanologenic host may include, *e.g.*, increasing the ability of organism to produce ethanol, depolymerize a particular substrate, tolerate a higher  
10 ethanol level, *etc.* In a preferred embodiment, the recombinant host is a Gram-negative bacterium. In another embodiment, the recombinant host is from the family Enterobacteriaceae. The ethanologenic hosts of U.S.P.N. 5,821,093, hereby incorporated by reference, for example, are suitable hosts and include, in particular, *E. coli* strains KO4 (ATCC 55123), KO11 (ATCC 55124), and KO12 (ATCC 55125). In  
15 addition, the LY01 strain may be employed as described in U.S.S.N. 08/834,900 and this application is hereby incorporated by reference.

It will be appreciated that host cells prepared according to the method of the invention may be used in conjunction with another recombinant host that expresses, yet another desirable polypeptide, *e.g.*, a different passenger gene sequence. In a particular  
20 example, a non-ethanologenic host cell may be used in conjunction with an ethanologenic host cell and either one or both of these host cells may be engineered according to the methods of the invention to accomplish, *e.g.*, one step of a multistep process, *e.g.*, the converting of a substrate into ethanol. For example, a non-ethanologenic host may be engineered for carrying out, *e.g.*, the depolymerization of a  
25 complex sugar followed by the use of an engineered ethanologenic host for fermenting the depolymerized sugar. Accordingly, it will be appreciated that the host cells of the invention may be used serially or contemporaneously for carrying out a particular process.

This invention is further illustrated by the following examples which should not  
30 be construed as limiting.

***EXEMPLIFICATION*****EXAMPLE I****5        Construction of Targeting Vectors for Introducing Heterologous DNA that is  
Excisable *In Vivo***

In this example, selectable targeting vectors for introducing heterologous DNA into a recipient host cell are described.

10        Throughout the example, the following materials and methods were used unless otherwise stated.

***Materials and Methods***

The bacterial strains and plasmids used in this example are listed in Table 1,  
15    below.

**Table 1. Bacterial strains and plasmids**

<i>E. coli</i> STRAINS	RELEVANT GENOTYPE	SOURCE or REFERENCE
DH5 $\alpha$	<i>lacZ</i> $\Delta$ M15 <i>recA1 endA1 hsdR17</i> [ <i>r<sub>K</sub><sup>-</sup>m<sub>K</sub><sup>+</sup></i> ] <i>supE44</i>	Promega
S17-1	<i>thi pro recA hsdR</i> RP4-2- <i>tet::Mu aphA::Tn7 Sp<sup>r</sup> Tp<sup>r</sup></i>	deLorenzo <i>et al.</i> , 1990
TOP10F <sup>+</sup>	F <sup>+</sup> { <i>lacI<sup>q</sup></i> Tn10(Tc <sup>r</sup> )} <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74 recA1 deoR araD139 <math>\Delta</math>(<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (St<sup>r</sup>) <i>endA1 nupG</i></i>	Invitrogen
SE2272	prototrophic strain B, $\Delta$ <i>frd102 zjd</i>	K. T. Shanmugan collection
SE2275	prototrophic strain K-12, $\Delta$ <i>frd102 zjd</i>	K. T. Shanmugan collection
FM7	SE2272 derivative containing pLOI2231 integrated (Km <sup>R</sup> , Cm <sup>R</sup> )	This study
FM19	SE2275 derivative containing pLOI2231 integrated (Km <sup>R</sup> , Cm <sup>R</sup> )	This study
FM18	FM7 derivative (Km <sup>S</sup> , Cm <sup>R</sup> )	This study
FM20	FM19 derivative (Km <sup>S</sup> , Cm <sup>R</sup> )	This study
LY01	<i>frd pfl::pdc adhB cat alc<sup>R</sup></i>	Yomano <i>et al.</i> , <i>J. Ind. Microbiol. Biotech.</i> 20:132-138 (1998)
PLASMIDS	DESCRIPTION	SOURCE OR REFERENCE



**Table 1. Continued**

pUC18	colE1ori; Ap <sup>R</sup>	Messing, J., <i>Methods Enzymol.</i> 101:20-78 (1983)
pCR2.1-TOPO	colE1ori; Ap <sup>R</sup> ; Km <sup>R</sup>	Invitrogen
LITMUS38	colE1ori; M13ori; Ap <sup>R</sup>	New England Biolabs, Inc.
pSG76A-K-C	$\gamma$ -R6Kori; FRT; Ap <sup>R</sup> -Km <sup>R</sup> -Cm <sup>R</sup>	Posfai <i>et al.</i> , 1997
pST76A-K-C	pSC101ori; FRT; Ap <sup>R</sup> -Km <sup>R</sup> -Cm <sup>R</sup>	Posfai <i>et al.</i> , 1997
PFT-A	pSC101ori; FLP gene under control of the tetracycline repressor; Ap <sup>R</sup>	Posfai <i>et al.</i> , 1997
pLOI2216	<i>XmnI/HindIII</i> fragment substitution from pSG76-A into pUC18; FRT1; colE1ori; Ap <sup>R</sup>	This study
pLOI2217	<i>HindIII</i> site deletion of pLOI2216; FRT1; colE1ori; Ap <sup>R</sup>	This study
pLOI2218	<i>EcoRI/PstI</i> fragment deletion of pLOI2217; FRT1; colE1ori; Ap <sup>R</sup>	This study
pLOI2219	<i>EcoRI</i> site substitution of <i>Clal</i> site into pLOI2218; FRT1; colE1ori; Ap <sup>R</sup>	This study
pLOI2220	<i>ScaI/EcoRI</i> fragment substitution from pLOI2401 into pLOI2219; FRT1; colE1ori; Ap <sup>R</sup>	This study
pLOI2221	<i>AscI</i> site substitution of <i>EcoRI</i> site into pLOI2220; FRT1; colE1ori; Ap <sup>R</sup>	This study
pLOI2222	<i>HindIII</i> site deletion of pLOI2221; FRT1; colE1ori; Ap <sup>R</sup>	This study
pLOI2223	<i>EcoRI/SphI</i> fragment substitution from pLOI2222 into pSG76-A; FRT1; FRT2; $\gamma$ -R6Kori; Ap <sup>R</sup>	This study
pLOI2224	<i>EcoRI/SphI</i> fragment substitution from pLOI2222 into pSG76-K; FRT1; FRT2; $\gamma$ -R6Kori; Km <sup>R</sup>	This study
pLOI2225	<i>EcoRI/SphI</i> fragment substitution from pLOI2222 into pSG76-C; FRT1; FRT2; $\gamma$ -R6Kori; Cm <sup>R</sup>	This study

**Table 1. Continued**

pLOI2226	<i>EcoRI/SphI</i> fragment substitution from pLOI2222 into pST76-A; FRT1; FRT2; pSC101ori; Ap <sup>R</sup>	This study
pLOI2227	<i>EcoRI/SphI</i> fragment substitution from pLOI2222 into pST76-K; FRT1; FRT2; pSC101ori; Km <sup>R</sup>	This study
pLOI2228	<i>EcoRI/SphI</i> fragment substitution from pLOI2222 into pST76-C; FRT1; FRT2; pSC101ori; Cm <sup>R</sup>	This study
pLOI2229	<i>EcoRI</i> fragment from pLOI2408 into pLOI2403; colE1ori; Ap <sup>R</sup> Km <sup>R</sup>	This study
pLOI2230	<i>BamHI</i> fragment from pLOI510 (Ohta <i>et al.</i> , 1991) into pLOI2229; colE1ori; Ap <sup>R</sup> , Km <sup>R</sup> , Cm <sup>R</sup>	This study
pLOI2231	<i>AscI</i> fragment from pLOI2230 into pLOI2224; $\gamma$ -R6Kori; Km <sup>R</sup> , Cm <sup>R</sup>	This study
pLOI2400	<i>AscI</i> site substitution of <i>SphI</i> site into pUC18; colE1ori; Ap <sup>R</sup>	This study
pLOI2401	<i>BglII</i> site substitution of <i>KpnI</i> site into pLOI2400; colE1ori; Ap <sup>R</sup>	This study
pLOI2402	<i>AscI</i> site substitution of <i>ApaI</i> site into LITMUS38; colE1ori; M13ori; Ap <sup>R</sup>	This study
pLOI2403	<i>AscI</i> site substitution of <i>StuI</i> site into pLOI2402; colE1ori; M13ori; Ap <sup>R</sup>	This study
pLOI2408	<i>adhE</i> gene into pCR2.1-TOPO; colE1ori; Ap <sup>R</sup> , Km <sup>R</sup>	This study
pLOI2413	<i>adhE</i> gene into pLOI2403; colE1ori; M13ori; Ap <sup>R</sup>	This study
pLOI510	promoterless <i>pdh adhB</i> genes from <i>Z. mobilis</i> and <i>cat</i> gene cloned into <i>BamHI</i> site of pUC18; colE1ori; Ap <sup>R</sup> , Cm <sup>R</sup>	Ohta <i>et al.</i> , 1991

For all plasmid constructions, standard methods were employed (Sambrook, J., *et al. Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY* (1989)). Reagents used in cloning were molecular biology grade and used as directed by the manufacturers. Restriction enzymes, T4 DNA polymerase, and Klenow polymerase were purchased from New England Biolabs (Beverly, MA). *Taq* PCR MasterKit was purchased from Qiagen (Santa Clarita, CA).

10 Polymerase chain reactions (PCR) were carried out using an Eppendorf Mastercycler (Westbury, NY). Primers were obtained from Genosys Biotechnologies (The Woodlands, TX). PCR products were ligated into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) using topoisomerase. DNA fragments were isolated from gels using a QIAquick

Gel Extraction Kit. DNA fragments were assembled using a Rapid DNA ligation Kit (Boehringer Mannheim Corporation, Indianapolis, IN). Wizard Plus kits (Promega, Madison, WI) were used for plasmid purification. Dideoxy sequencing of plasmids was performed by using fluorescent primers and a LI-COR model 4000L DNA Sequencer  
5 (Lincoln, NB) as previously described (Lai, X., *et al.*, *Appl. Envir. Microbiol.* 63:355-363 (1996)). Except where mentioned, all ligation reactions were done with the Rapid DNA Ligation Kit and used to transform *E. coli* DH5 $\alpha$ , followed by selection in LB plates containing ampicillin.

#### 10 *Plasmid Constructions*

Two sets of insertion vectors were constructed using, as an initial vector, the suicide plasmids pSG76 and pST76 series, described by Posfai *et al.* (1997). These low copy number plasmids contain the conditional replicons R6K- $\gamma$  and pSC101, respectively. Modifications were executed in several steps so that another recombining  
15 site, *i.e.*, FRT site, was introduced into each of original vectors, as well as a more suitable multiple cloning site (MCS). Unless mentioned in the text, all new plasmids are shown in brackets, just after its corresponding description. Complete sequences for the following plasmids have been deposited in GenBank with the following accession numbers: pLOI2223, AF172933; pLOI224, AF172934; pLOI225, AF172935; pLOI226,  
20 AF172936; pLOI227, AF172937; pLOI228, AF172938 (see also, respectively, SEQ ID NOS: 1-6).

To facilitate the manipulation of each construct, most of the plasmid manipulations were done in pUC18 (Messing, J., *Methods Enzymol.* 101:20-78 (1983)) and derivatives made herein. An 800 bp *Xmn*I-*Hind*III fragment from pUC18 was  
25 exchanged for a fragment comprising part of the *bla* gene plus the FRT site from pSG76-A to create pLOI2216. Digestion, Klenow treatment and self-ligation [pLOI2217] abolished the *Hind*III site in pLOI2216. The restriction sites from *Eco*RI to *Pst*I were removed by double-digestion with these enzymes, *Klenow* treatment and self-ligation [pLOI2218]. Then a new *Eco*RI site was created by linker addition into the  
30 previously added *Cla*I site, giving rise to a pUC18- plasmid derivative carrying one FRT site (called FRT1 in Table 1) but no MCS [pLOI2219].

To introduce a new MCS into pLOI2219, the *SphI* site from pUC18 was replaced with an *AscI* site by linker addition [pLOI2400], and the *KpnI* site was replaced with a *BglII* linker [pLOI2401]. The modified pUC18-based MCS was then introduced into pLOI2219 as a 965 bp *ScaI/EcoRI* fragment originating from pLOI2220. By linker  
5 addition, an *AscI* site took the place of *EcoRI* site [pLOI2221], and a new *EcoRI* site replaced the *HindIII* site [pLOI2222].

The resultant plasmid was sequenced in both direction and used as a source of DNA carrying the modified MCS and the FRT1 site.

#### 10 *Construction of pLOI2223*

To create the conditional replication vector carrying two FRT sites and the ampicillin-resistance gene, pLOI2222 was double-digested with *EcoRI* and *ScaI* and a 157 bp fragment was ligated to pSG76-A (R6K- $\gamma$  *ori*, Ap<sup>R</sup>) digested with the same enzymes. Transformation was done in *E. coli* S17-1, since this strain provides the  $\lambda$ *pir*  
15 protein involved in the replication of the R6K- $\gamma$  *ori* and selection was done in the presence of 50  $\mu$ g/ml ampicillin. The resultant plasmid was denominated pLOI2223.

#### *Construction of pLOI2224*

To create the conditional replication vector carrying two FRT sites and the  
20 kanamycin-resistance gene, pLOI2222 was double-digested with *EcoRI* and *ScaI* and a 157 bp fragment was ligated to pSG76-K (R6K- $\gamma$  *ori*, Km<sup>R</sup>) digested with the same enzymes. Transformation was done in *E. coli* S17-1, since this strain provides the  $\lambda$ *pir* protein involved in the replication of the R6K- $\gamma$  *ori* and selection was done in the presence of 50  $\mu$ g/ml kanamycin. DNA from several colonies was prepared and  
25 analyzed by digestion with *AscI* and *BglII*, separately, to confirm the presence of the modified MCS. The resultant plasmid was denominated pLOI2224.

#### *Construction of pLOI2225*

To create the conditional replication vector carrying two FRT sites and the  
30 chloramphenicol-resistance gene, pLOI2222 was double-digested with *EcoRI* and *ScaI* and a 157 bp fragment was ligated to pSG76-C (R6K- $\gamma$  *ori*, Cm<sup>R</sup>) digested with the same enzymes. Transformation was performed as described above and selection was done in

the presence of 50 µg/ml chloramphenicol. The resultant plasmid was termed pLOI2225.

#### *Construction of pLOI2226*

- 5           To create the thermosensitive replication vector carrying two FRT sites and the ampicillin-resistance gene, pLOI2222 was double-digested with *EcoRI* and *ScaI* and a 157 bp fragment was ligated to pST76-A (pSC101 *ori*, Ap<sup>R</sup>) digested with the same enzymes. For plasmids carrying the thermosensitive replicon (pSC101), *E. coli* S17-1 transformed cells were kept at 30°C and cells were selected with 40 µg/ml ampicillin.
- 10   The resultant plasmid was termed pLOI2226.

#### *Construction of pLOI2227*

- To create the thermosensitive replication vector carrying two FRT sites and the kanamycin-resistance gene, pLOI2222 was double-digested with *EcoRI* and *ScaI* and a
- 15   157 bp fragment was ligated to pST76-K (pSC101 *ori*, Km<sup>R</sup>) digested with the same enzymes and transformation was performed as described above and selection was done in the presence of 40µg/ml of kanamycin. The resultant plasmid was termed pLOI2227.

#### *Construction of pLOI2228*

- 20           To create the thermosensitive replication vector carrying two FRT sites and the chloramphenicol-resistance gene, pLOI2222 was double-digested with *EcoRI* and *ScaI* and a 157 bp fragment was ligated to pST76-C (pSC101 *ori*, Cm<sup>R</sup>) digested with the same enzymes and transformation was performed as described above and selection was done in the presence of 40µg/ml of chloramphenicol. The resultant plasmid was termed
- 25   pLOI2228.

#### *Construction of pLOI2231*

- The construct pLOI2231 (SEQ ID NO: 7) is a derivative of pLOI2224 where *adhE* (guide) and the PET (passenger) genes have been cloned. The PET passenger
- 30   genes comprise *adhB* and *pdC*. To obtain this construction the *adhE* gene was first cloned into pCR2.1-TOPO vector as indicated by manufacturer. Briefly, the *adhE* gene was PCR amplified using a pair of Genosys ORFmer primers:

5 'TTGCTCTTCCATGGCTGTTACTAATGTCGCTGAA3' forward primer;

5 'TTGCTCTTCGTTAAGCGGATTTTTTCGCTTTTTTCT3' reverse primer. The PCR

product of *adhE* includes the initiation codon ATG and the termination codon TAA. A fresh PCR sample of *adhE* was ligated to the pCR2.1-TOPO vector. This plasmid was

5 designated as pLOI2408. A subcloning step was performed to transfer *adhE* from pLOI2408 to pLOI2403. Plasmid pLOI2408 was digested with restriction enzyme *EcoRI*. The product of this digestion allows to obtain the whole PCR product previously cloned. The *adhE* fragment was separated by gel electrophoresis and isolated from the gel using the QIAGEN kit.

10 The isolated *adhE* fragment was visualized by UV light after ethidium bromide staining and gel electrophoresis. This fragment was ligated to plasmid pLOI2403. The construct pLOI2403 was previously digested with restriction enzyme *EcoRI*. The *adhE* gene fragment and pLOI2403 were ligated using the rapid DNA ligation kit as directed by manufacturer. The plasmid obtained after the ligation between the *adhE EcoRI*  
15 digested fragment and pLOI2403 *EcoRI* digested plasmid was designated pLOI2413. In this plasmid, the 3' end of the *adhE* gene is orientated to be close to the *BamHI* site of pLOI2403 as revealed by sequence analysis.

In order to ligate the PET genes (passenger) to *adhE* gene (guide) a ligation was performed between a PET fragment and the plasmid pLOI2413. PET genes were  
20 isolated from plasmid pLOI510 as a *BamHI* fragment of about 4.4 Kbp. The PET *EcoRI* digested fragment was isolated from a gel after electrophoresis of plasmid pLOI510 *BamHI* digested with the QIAGEN kit. The PET DNA fragment was visualized by ethidium bromide after gel electrophoresis. A ligation of the isolated PET fragment with plasmid pLOI2413 previously digested with restriction enzyme *BamHI* was performed.  
25 The plasmid obtained was designated pLOI2230.

To obtain an integration vector with guide and the passenger fragments as shown in Fig. 3, the plasmid pLOI2224 was ligated to a fragment containing *adhE* and PET genes isolated from plasmid pLOI2230. The resultant plasmid termed pLOI2230 was digested with the restriction enzyme *AscI* and the digestion product was cloned into  
30 pLOI2224 previously digested with enzyme *AscI*. The plasmid obtained from this ligation was designated pLOI2231 and the orientation of the various genetic elements of the plasmid are shown in Fig. 3

### Targeting Strategy

Recombinase-based integration systems offer the opportunity to effect precise DNA deletions *in vivo* (Cherepanov, P. P., *et al. Gene* 158: 9-14 (1995); Posfai, G. *et al., Nucleic Acids Res.* 22: 2392-2398 (1994)) and *in vitro* (Cox, M. M., *Proc. Natl. Acad. Sci. USA* 80: 4223-4227 (1983); Hasan, N., *et al., Gene* 150: 51-56 (1994), Wild, J., Z. *et al., Gene* 179:181-188 (1996)). The present invention encompasses plasmids as described by Posfai *et al. (J. Bacteriol.* 179: 4426-4428 (1997)) that were further modified to incorporate homologous DNA as a guide sequence, two FRT sites in the same orientation flanking an antibiotic marker and a multi cloning site (MCS) (Fig. 1).

A full set of plasmids (ampicillin, chloramphenicol, and kanamycin resistance) was made for each conditional replicon (Fig. 1; pSC101 and R6K- $\gamma$ ). Since both conditional replicons are present at low copy numbers, an additional high copy vector was developed to facilitate constructions by adding an *AscI* site on either side of the MCS in pLITMUS38 (New England Biolabs, Beverly, MA) to produce pLOI2403 (Fig. 1).

A general procedure for chromosomal integration of DNA using the new vectors with conditional replicons is presented in Fig. 2. The integration of heterologous passenger DNA carrying desired functions can be targeted to any specific chromosomal site by an adjacent fragment of homologous DNA (guide) during growth at 30°C (pSC101), or directly selected at 37-42°C (pSC101 or R6K- $\gamma$ ). With a single cross-over event, the entire plasmid is incorporated into the chromosome (If needed, pSC101-based integration vectors can be eliminated by overnight growth and plating at elevated temperatures.).

After integration, recombinants were transformed with pFT-A containing the yeast FLP gene under control of the tetracycline promoter and grown under permissive conditions (30°C, pSC101). During growth with chlortetracycline, FLP recombinase was induced which in turn resulted in the excision of the DNA bracketed by the concurrently-facing *FRT* sites (selectable marker and replicon) from the chromosome as shown in Fig. 3. After growth at 37-42°C to eliminate plasmid pFT-A encoding the recombinase, only the passenger gene(s), a single *FRT*, and the homologous guide fragment remained in the chromosome as described in further detail below.

**EXAMPLE II****A Method for Engineering an Ethanologenic Host Cell by Chromosomal Integration of Heterologous DNA**

5

In this example, methods for introducing heterologous DNA to engineer an ethanologenic organism are described.

To illustrate the utility of the foregoing vectors, heterologous genes were targeted into the genome of two different test microorganism in order to generate a genetically engineered organism with a desired phenotype (*i.e.*, ability to produce ethanol). In particular, derivatives of *E. coli* B (strain SE2272,  $\Delta frd$ ) and *E. coli* K-12 (SE2275,  $\Delta frd$ ) were constructed in which three heterologous genes were integrated immediately behind the endogenous *adhE* gene in the chromosome. The guide and passenger DNA were cloned into pLOI2403, a high copy plasmid vector described above. For this construction, the promoterless *adhE* coding region (guide) was amplified with Genosys ORFmer primers for the coding region (forward, 5' TTGCTCTTCCATGGCTGTTACTAATGTCGCTGAA3' ; reverse, 5' TTGCTCTTCGTTAAGCGGATTTTTTCGCTTTTTTCT3' ) and cloned into pCR2.1-TOPO to produce pLOI2408 (see for example Fig. 3). After *Eco*RI digestion, the 2.6 Kbp *adhE* region from pLOI2408 was moved into the corresponding site in pLOI2403 to produce pLOI2413. The *Bam*HI site immediately downstream from the 3' end of the *adhE* coding region was used to insert a 4.6 Kbp *Bam*HI fragment from pLOI510 (Ohta, K. *et al.*, *Appl. Environ. Microbiol.* 57: 893-900 (1991)) containing three genes (passenger): a promoterless *Zymomonas mobilis pdc* without transcriptional terminator and a promoterless *Z. mobilis adhB* with a transcriptional terminator, followed by a complete *cat* operon with promoter and terminator. In the resulting plasmid (pLOI2230), transcription of the heterologous genes was oriented concurrently with *adhE*. All constructs containing the *Z. mobilis* genes were grown in LB supplemented with glucose (20 g/L for plates; 50 g/L for broth).

30 The 7.2 Kbp *Asc*I fragment from pLOI2230 (high copy vector) containing *adhE*, the artificial operon *pdc adhB*, and *cat* was ligated into the low copy integration vector pLOI2224 which contains a R6K- $\gamma$  replicon ( $\lambda$ *pir*-dependent) and transformed into the



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permissive host S17-1 (de Lorenzo, V., *et al.*, *J. Bacteriol.* 172: 6568-6572 (1990)) with selection for kanamycin and chloramphenicol. The resulting clone containing pLOI2231 was used for large-scale plasmid isolation (500 ml) by the alkaline lysis procedure (Sambrook, J., *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)).

Approximately 500 ng of pLOI2231 DNA was used for electroporation of SE2272 and SE2275. Both are non-permissive hosts. Recombinants were readily obtained by selection for either kanamycin (vector) or chloramphenicol (passenger) resistance. Up to 2 h was allowed for expression of the resistance gene prior to spreading on plates for selection. Approximately 1000 recombinants per 1 µg DNA (electroporation) were recovered using *E. coli* K-12 SE2275, 5-fold higher than that obtained with the *E. coli* B SE2272. Thirty recombinants from each host were screened for the functional expression of alcohol dehydrogenase on indicator plates (Conway, T.C., *et al.*, *J. Bacteriol.* 169:2591-2597 (1987)). Based on the rate and intensity of color development, these recombinants expressed higher levels of ADH activity than the respective unmodified SE2272 or SE2275, or S17-1(pLOI2231) harboring promoterless *pdh* and *adhB* genes. Unlike the control strains, these recombinants also exhibited a colonial phenotype (large raised colonies on LB containing glucose) that is typical for ethanologenic *E. coli* (Ingram, L.O., *et al.*, *Environ. Microbiol.* 53: 2420-2425 (1987)). Small-scale DNA preparations (7 recombinants per host) were tested for the presence of pLOI2231. None contained plasmids as tested by gel filtration or based on transformation experiments using S17-1 as the host. These recombinants were presumed to contain chromosomally integrated genes. One clone from each parent, strains FM7 (*E. coli* B SE2272) and FM19 (*E. coli* K-12 SE2275), was selected for further study.

Accordingly, strains FM7 and FM19 were transformed with the helper plasmid (pFT-A) carrying the FLP gene (Posfai, G., *et al.*, *J. Bacteriol.* 179: 4426-4428 (1997)) and incubated at 30°C with selection for ampicillin resistance. A mixture of colonies was used to inoculate a broth culture for induction of FLP with autoclaved chlortetracycline (20 µg/ml). After 6 h incubation at 30°C, the culture was diluted 1:1000 in LB containing glucose and incubated at 42°C for 16 h to eliminate the helper plasmid. After streaking on solid medium, isolated colonies were screened for the

absence of antibiotic markers. Approximately 80% of the colonies were ampicillin and kanamycin sensitive and retained only chloramphenicol resistance and the ethanologenic traits (passenger genes inserted into the MCS). Loss of ampicillin resistance indicated that the helper plasmid had been successfully eliminated while loss of kanamycin resistance confirmed the FLP-recombinase-dependent deletion of the vector. These new derivatives of FM7 and FM19 were designated FM18 and FM20, respectively.

PCR was used to verify the integration events in both FM18 and FM20. Two new sets of primers were designed to amplify the *adhE* gene including the unique junctions predicted for *pdv* (Fig. 3, primers 3 and 4) and *cat* (Fig. 3, primers 5 and 6) as a result of integration and FLP-mediated deletion. Forward primer 3 hybridizes to the promoter region of *adhE* while reverse primer 4 hybridizes to the N-terminal coding region of *pdv*. Forward primer 5 hybridizes to the C-terminal coding region of *cat* gene and reverse primer 6 hybridizes to the 3' untranslated portion of the *adhE*. Note that the primers used to clone *adhE*, forward primer 1 and reverse primer 2, hybridize to the terminal and C-terminal coding regions of *adhE* and are inside of the regions encoded by forward primer 3 and reverse primer 6. All primer sets (SE2272 template: 1+2, 3+6; FM18 template: 3+4, 5+6) generated products of the expected sizes (Fig. 4A). Identical results were also obtained using FM20 DNA as the template. As shown in Fig. 3, and arrows with numbers 1, 2, 3, 4, 5, and 6 represent primers used to amplify the corresponding regions. The sequences for these primers are as follows: Primer 1. 5' TTGCTCTTCCATGGCTGTTACTAATGTCGCTGAA3'; Primer 2. 5' TTGCTCTTCGTTAAGCGGATTTTTTCGCTTTTTTCT3'; Primer 3. 5' GTGAGTGTGAGCGCGGAGT3'; Primer 4. 5' TGGCACGAGCATAACCTTC3'; Primer 5. 5' CAGTACTGCGATGAGTGGCA3'; Primer 6. 5' GTTGCCAGACAGCGCTACT3'.

The *adhE* gene contains a single central *BstEII* site which does not occur elsewhere in the PCR products. This site was used to verify the identity of the PCR fragments. As shown in Fig. 4B, all PCR products were cut once to produce fragments containing the N-terminal and C-terminal regions of *adhE*. Fragments from the *adhE* coding region alone (primers 1+2) were smaller (N-terminal fragment = 1,226 bp; C-terminal fragment = 1,470 bp) than fragments which included parts of the native *adhE* promoter (primers 3+4 and 3+6; N-terminal fragment = 1,325 bp) or *adhE* terminator (primers 3+6, 5+6; C-terminal fragment = 1,489 bp). The fragment which included part

of *pdc* (primers 3+4) was the largest C-terminal fragment (1,783 bp). The fragment which included part of *cat* (primers 5+6) was the largest N-terminal fragment (1,988 bp).

In Fig. 4, panel A shows full length PCR products of *adhE* junctions. Positions and sequences of primers are provided in Fig. 3. The lanes in panel A of Fig. 4  
5 represent: 1., *Hind*III digest of phage  $\lambda$  DNA; 2., *adhE* coding region (2,696 bp) of SE2272 amplified using forward primer 1 and reverse primer 2; 3., *adhE* promoter and 3' untranslated sequence (2,814 bp) of SE2272 amplified using forward primer 3 and reverse primer 4; 4., The *adhE* and *pdc* junction (3,108 bp) of FM18 amplified using forward primer 3 and reverse primer 4; 5., The *cat* junction and *adhE* (3,477 bp) of  
10 FM18 amplified using forward primer 5 and reverse primer 6. The amplification conditions for primer pairs 1 + 2 and 3 + 6 (25 cycles) were: 45 sec at 94°C, 45 sec at 60°C, 60 sec at 72°C. The amplification conditions for primer pairs 3 + 4 and 5 + 6 (30 cycles) were: 45 sec at 94°C, 60 sec at 60°C. DNA was held at 94°C for 3 min prior to the first cycle. The elongation time was increased to 10 min during the final cycle.

15 In Fig. 4, panel B shows a *Bst*EII digestion of the above-described PCR products. A single, central *Bst*EII site was used to cleave PCR products containing *adhE* into a N-terminal and C-terminal fragments. The lanes in panel B of Fig. 4 show the following: Lane 1 contains a DNA standard (descending: 3.0, 2.0, 1.5, 1.2, 1.0 Kbp); Lanes 2 through 5 contain *Bst*EII digested PCR products of fragments described in 4A,  
20 respectively.

The expression of *adhE* is regulated by a number of factors in *E. coli* including *cra*, *adhR*, and the abundance of NADH (Leonardo, M.R., *et al.*, *J. Bacteriol.* 175: 870-878 (1993); Leonardo, M.R., *et al.*, *J. Bacteriol.* 178: 6013-6018 (1996); Mikulskis, A., *et al.*, *J. Bacteriol.* 179: 7129-7134 (1997)). Both message levels and activity are  
25 approximately 10-fold higher during anaerobic growth with glucose than during aerobic growth. Since the *Z. mobilis* genes are integrated behind the *adhE* coding region to form an operon fusion, expression of *pdc* should also increase in response to anaerobiosis. Strains FM18 and FM20 were grown in Luria broth containing 50 g glucose/liter under aerobic and anaerobic conditions. PDC activities were determined in heat-treated  
30 preparations to eliminate lactate dehydrogenase and other confounding activities (Conway, T., *et al.*, *J. Bacteriol.* 169: 949-954 (1987)). Under anaerobic conditions, PDC activities in FM18 and FM20 were 0.254 U/mg protein and 0.185 U/mg protein,

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respectively, approximately 4-fold higher than the activities observed in cells grown under aerobic conditions.

The above results demonstrate that the new integration vectors can be used to place promoterless genes under the control of a chromosomal promoter in a site specific fashion. Moreover, after the integration event, unnecessary nucleic acid encoding a replicon and selectable marker was removed *in vivo* using a recombining site specific recombinase. This approach avoids potential problems of lethality or mutation due to unregulated expression in plasmids during construction and integration. These vectors can also be used to replace promoters in chromosomal genes. Additional unique restriction sites are available for the insertion of genes which can be temporarily expressed after integration and subsequently deleted with the replicon and selectable marker. This option, the temporary introduction of new genes, may be useful to test new traits in an isogenic background.

Although the vectors described must be propagated in *E. coli*, they are potentially useful with other organisms. The FLP recombinase is extremely efficient (Wild, J., Z., *et al.*, *Gene* 179:181-188 (1996)) and could be produced intracellularly as a transient expression product after transformation or electroporation of pFT-A.

### ***Equivalents***

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. Moreover, any number of genetic constructs, host cells, and methods described in United States Patent Nos. 5,821,093; 5,482,846; 5,424,202; 5,028,539; 5,000,000; 5,162,516; and U.S. patent application serial No. 60/136,376 may be employed in carrying out the present invention and are hereby incorporated by reference.

**Claims**

1. A method for integrating a nucleic acid construct into the genome of a host cell, said method comprising,
  - 5 contacting said cell with a nucleic acid construct under conditions such that said nucleic acid construct is integrated by said cell, wherein said nucleic acid construct comprises a passenger sequence and a marker sequence, wherein said marker sequence is flanked by a first and second recombining site wherein said recombining sites are oriented in the same direction.
- 10 2. The method of claim 1, wherein said construct further comprises an origin of replication between said first and second recombining sites.
3. The method of claim 2, wherein said origin of replication is, or is derived from,
  - 15 an origin of replication selected from the group consisting of pSC101ori, R6K-γori, colE1, and oriEV.
4. The method of claim 1, wherein said nucleic acid further comprises a sequence selected from the group consisting of a promoter, a restriction site, an intron, an IRES
  - 20 element, and a polyadenylation site.
5. The method of claim 1, wherein said nucleic acid further comprises a guide sequence thereby resulting in site-specific integration of said nucleic acid construct.
- 25 6. The method of claim 1, wherein said guide sequence, comprises genomic sequence derived from a replicating genome.
7. The method of claim 1, wherein said cell is provided with a site-specific recombinase that results in recombination between said first and second recombining
  - 30 sites, wherein said sequence flanked by said recombining sites is excised.

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8. The method of claim 7, wherein said recombinase is selected from the group consisting of FLP, Xer, Int, and Cre.
9. The method of claim 7, wherein said recombinase is FLP recombinase.
- 5 10. The method of claim 1, wherein said recombining site is selected from the group consisting of FRT, dif, att, and loxP.
11. The method of claim 1, wherein said recombining site is FRT.
- 10 12. The method of claim 7, wherein following excision of said sequence, the method of claim 1 is repeated on said cell.
13. The method of claim 1, wherein said construct further comprises a promoter 5' to  
15 the passenger sequence.
14. The method of claim 1, wherein said marker sequence is selected from the group consisting of an antibiotic resistance gene and a non-antibiotic resistance gene.
- 20 15. The method of claim 14, wherein said antibiotic resistance gene is selected from the group consisting of the gentamycin resistance gene, zeocin resistance gene, kanamycin resistance gene, ampicillin resistance gene, tetracycline resistance gene, and chloramphenicol resistance gene.
- 25 16. The method of claim 11, wherein said non-antibiotic resistance gene is selected from the group consisting of an auxotrophic gene, a metal ion resistance gene, a *trp* gene, and a gene encoding a green fluorescent protein.
17. The method of claim 1, wherein said passenger sequence encodes at least one  
30 gene.

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18. The method of claim 17, wherein said passenger sequence encodes a gene involved in ethanogenesis.

19. The method of claim 18, wherein said gene involved in ethanogenesis is  
5 derived from an organism selected from the group consisting of prokaryotes and eukaryotes.

20. The method of claim 18, wherein said gene is selected from the group consisting of *adh* and *pdc*.

10

21. The method of claim 1, wherein said host cell is a bacterial cell.

22. The method of claim 1, wherein said host cell is a Gram-negative bacterial cell.

15 23. The method of claim 22, wherein said host cell is a facultatively anaerobic bacterial cell.

24. The method of claim 23, wherein said host cell is selected from the family Enterobacteriaceae.

20

25. The method of claim 24 wherein said host is selected from the group consisting of *Escherichia* and *Klebsiella*.

26. The method of claim 24, wherein said host is *E. coli* B or *E. coli* K12.

25

27. The method of claim 1, wherein said host cell is a recombinant bacterial cell.

28. The method of claim 1, wherein said nucleic acid construct is, or is derived from, a plasmid selected from the group consisting of pLOI2223, pLOI2224, pLOI2225,  
30 pLOI2226, pLOI2227, pLOI2228, pLOI2231, and pLOI2403.

29. A method for producing a recombinant ethanologenic cell, said method comprising,

contacting said cell with a nucleic acid construct under conditions in which integration of said nucleic acid construct occurs forming a recombinant ethanologenic cell, wherein said nucleic acid construct comprises a passenger sequence, wherein said passenger sequence comprises an ethanologenic gene, and a marker sequence, wherein said marker sequence is flanked by a first and second recombining site.

30. The method of claim 29, wherein said construct further comprises an origin of replication between said first and second recombining site.

31. The method of claim 30, wherein said origin of replication is, or is derived from, an origin of replication selected from the group consisting of pSC101ori, R6K- $\gamma$ ori, colE1ori, and oriEV.

15

32. The method of claim 29, wherein said cell is provided with a site-specific recombinase that results in recombination between said first and second recombining sites wherein said sequence flanked by said recombining sites is excised.

20 33. The method of claim 29, wherein said recombinase is selected from the group consisting of FLP, Xer, Int, and Cre.

34. The method of claim 33, wherein said recombinase is FLP recombinase.

25 35. The method of claim 29, wherein said recombining site is selected from the group consisting of FRT, dif, att, and loxP.

36. The method of claim 35, wherein said recombining site is FRT.

30 37. The method of claim 29, wherein said ethanologenic gene is selected from the group consisting of *adh* and *pdc*.



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38. The method of claim 29 where said passenger sequence encodes *pdh*, *adhB*, and *cat*.
39. The method of claim 29, wherein said nucleic acid further comprises a guide  
5 sequence thereby resulting in site-specific integration of said nucleic acid construct.
40. The method of claim 39, wherein said guide sequence is derived from a replicating genome.
- 10 41. The method of claim 29, wherein said marker sequence is selected from the group consisting of a antibiotic resistance gene or a non-antibiotic resistance gene.
42. The method of claim 41, wherein said antibiotic resistance gene is selected from the group consisting of gentamycin resistance gene, zeocin resistance gene, kanamycin  
15 resistance gene, ampicillin resistance gene, tetracycline resistance gene, and chloramphenicol resistance gene.
43. The method of claim 41, wherein said non-antibiotic resistance gene is selected from the group consisting of an auxotrophic gene, a metal ion resistance gene, a *trp*  
20 gene, and a gene encoding a green fluorescent protein.
44. The method of claim 41, where said marker sequence comprises the kanamycin resistance gene.
- 25 45. A recombinant ethanologenic cell produced according to the method of claim 29.
46. A recombinant host cell comprising,  
a nucleic acid construct that comprises a passenger sequence and a  
marker sequence, wherein said marker sequence is flanked by a first and second  
30 recombining site wherein said recombining sites are oriented in the same direction.

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47. The host cell according to 46, wherein said construct further comprises an origin of replication between said first and second recombining sites.

48. The host cell according to 46, wherein said origin of replication is, or is derived from, an origin of replication selected from the group consisting of pSC101ori, R6K-  
5 yori, colE1, and oriEV.

49. The host cell according to claim 46, wherein said nucleic acid construct is integrated.

10

50. The host cell according to claim 46, wherein said nucleic acid further comprises a guide sequence thereby resulting in site-specific integration of said nucleic acid construct.

15 51. The host cell according to claim 46, wherein said guide sequence, comprises genomic sequence derived from a replicating genome.

52. The host cell according to claim 46, wherein said cell is provided with a site-specific recombinase that results in recombination between said first and second  
20 recombining sites, wherein said sequence flanked by said recombining sites is excised.

53. The host cell according to claim 46, wherein said recombinase is selected from the group consisting of FLP, Xer, Int, and Cre.

25 54. The host cell according to claim 46, wherein said recombinase is FLP recombinase.

55. The host cell according to claim 46, wherein said recombining site is selected from the group consisting of FRT, dif, att, and loxP.

30

56. The host cell according to claim 46, wherein said recombining site is FRT.

57. The host cell according to claim 46, wherein said marker sequence is selected from the group consisting of a antibiotic resistance gene or a non-antibiotic resistance gene.

5 58. The host cell according to claim 57, wherein said antibiotic resistance gene is selected from the group consisting of a gentamycin resistance gene, zeocin resistance gene, kanamycin resistance gene, ampicillin resistance gene, tetracycline resistance gene, and chloramphenicol resistance gene.

10 59. The host cell according to claim 57, wherein said non-antibiotic resistance gene is selected from the group consisting of an auxotrophic gene, a metal ion resistance gene, a *trp* gene, and a gene encoding a green fluorescent protein.

60. The host cell according to claim 46, wherein said passenger sequence encodes a  
15 gene involved in ethanologensis.

61. The host cell according to claim 46, wherein said passenger sequence is selected from the group comprising *adh*, *pdc*, and *cat*.

20 62. The host cell according to claim 46, wherein said host cell is a bacterial cell.

63. The host cell according to claim 46, wherein said host cell is a Gram-negative bacterial cell.

25 64. The host cell according to claim 46, wherein said host cell is a facultatively anaerobic bacterial cell.

65. The host cell according to claim 46, wherein said host cell is selected from the family Enterobacteriaceae.

30

66. The host cell according to claim 46, wherein said host is selected from the group consisting of *Escherichia* and *Klebsiella*.

67. The host cell according to claim 46, wherein said host is *E. coli* B or *E. coli* K12.
68. The host cell according to claim 46, wherein said host cell is a recombinant bacterial cell.
- 5 69. The host cell according to claim 46, wherein said nucleic acid construct is, or is derived from, a plasmid selected from the group consisting of pLOI2223, pLOI2224, pLOI2225, pLOI2226, pLOI2227, pLOI2228, pLOI2231, and pLOI2403.
- 10 70. The host cell according to claim 46, wherein said cell is ethanologenic.
71. A method for producing ethanol comprising,  
providing a recombinant ethanologenic cell having a nucleic acid  
construct that comprises a passenger sequence and a marker sequence, wherein said  
15 marker sequence is flanked by a first and second recombining site, and  
contacting said cell with a substrate which can be fermented into  
ethanol, wherein expression of said passenger sequence results in the production of  
ethanol.
- 20 72. The method of claim 71, wherein said construct further comprises an origin of replication between said first and second recombining sites.
73. The method of claim 72, wherein said origin of replication is, or is derived from, an origin of replication selected from the group consisting of pSC101ori, R6K- $\gamma$ ori,  
25 colE1, and oriEV.
74. The method according to claim 71, wherein said nucleic acid construct is integrated.
- 30 75. The method according to claim 71, wherein said nucleic acid construct further comprises a guide sequence thereby resulting in site-specific integration of said nucleic acid construct.

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76. The method according to claim 75, wherein said guide sequence, comprises genomic sequence derived from a replicating genome.

77. The method according to claim 71, wherein said cell is provided with a site-specific recombinase that results in recombination between said first and second  
5 recombining site, wherein said sequence flanked by said recombining sites is excised.

78. The method according to claim 71, wherein said recombinase is selected from the group consisting of FLP, Xer, Int, and Cre.

10

79. The method according to claim 71, wherein said recombinase is FLP recombinase.

80. The method according to claim 71, wherein said recombining site is selected  
15 from the group consisting of FRT, dif, att, and loxP.

81. The method according to claim 71, wherein said recombining site is FRT.

82. The method according to claim 71, wherein said marker sequence is selected  
20 from the group consisting of a antibiotic resistance gene or a non-antibiotic resistance gene.

83. The method of claim 82, wherein said antibiotic marker is selected from the group consisting of a gentamycin resistance gene, zeocin resistance gene, kanamycin  
25 resistance gene, ampicillin resistance gene, tetracycline resistance gene, and chloramphenicol resistance gene.

84. The method of claim 82, wherein said non-antibiotic resistance gene is selected from the group consisting of an auxotrophic gene, a metal ion resistance gene, a *trp*  
30 gene, and a gene encoding a green fluorescent protein.

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85. The method according to claim 71, wherein said passenger sequence encodes a gene involved in ethanologensis.

86. The method according to claim 71, wherein said passenger sequence is selected  
5 from the group comprising *adh* and *pdh*.

87. The method according to claim 71, wherein said passenger sequence encodes *adhB*, *pdh*, and *cat*.

10 88. The method according to claim 71, wherein said host cell is a bacterial cell.

89. The method according to claim 71, wherein said host cell is a Gram-negative bacterial cell.

15 90. The method according to claim 71, wherein said host cell is a facultatively anaerobic bacterial cell.

91. The method according to claim 71, wherein said host cell is selected from the family Enterobacteriaceae.

20

92. The method according to claim 71, wherein said host is selected from the group consisting of *Escherichia* and *Klebsiella*.

93. The method according to claim 71, wherein said host is *E. coli* B or *E. coli* K12.

25

94. The method according to claim 71, wherein said host cell is a recombinant bacterial cell.

95. The method according to claim 71, wherein said nucleic acid construct is, or is  
30 derived from, a plasmid selected from the group consisting of pLOI2223, pLOI2224, pLOI2225, pLOI2226, pLOI2227, pLOI2228, pLOI2231, and pLOI2403.

- 45 -

96. A nucleic acid construct comprising,  
a passenger sequence, and  
a marker sequence, wherein said marker sequence is flanked by a first  
and second recombining site.

5

97. The construct according to claim 96, wherein said construct further comprises an  
origin of replication between said first and second recombining sites.

98. The construct according to claim 97, wherein said origin of replication is, or is  
10 derived from, an origin of replication selected from the group consisting of pSC101ori,  
R6K- $\gamma$ ori, colE1, and oriEV.

99. The construct according to claim 84, wherein said construct further comprises a  
guide sequence.

15

100. The construct according to claim 99, wherein said guide sequence comprising a  
sequence derived from a replicating genome.

101. The construct according to claim 99, wherein said guide sequence is derived  
20 from a bacterial cell.

102. The construct according to claim 96, wherein said construct further comprises a  
at least one unique restriction enzyme site.

25 103. The construct according to claim 96, wherein said passenger sequence comprises  
an ethanologenic gene.

104. The construct according to claim 96, wherein said passenger sequence comprises  
a gene selected from the group consisting of *adh* and *pdh*.

30

105. The construct according to claim 96, wherein said passenger sequence comprises  
*adhB*, *pdh*, and *cat*.

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106. The construct according to claim 96, wherein said recombining site is selected from the group consisting of FRT, dif, att, and loxP.

107. The construct according to claim 96, wherein said recombining site is FRT.

5

108. The construct according to claim 96, wherein said construct is exposed to a site-specific recombinase such that a recombination between said first and second recombining site occurs, wherein said sequence flanked by said recombining sites is excised.

10

109. The construct according to claim 108, wherein said recombinase is selected from the group consisting of FLP, Xer, Int, and Cre.

110. The construct according to claim 109, wherein said recombinase is FLP.

15

111. The construct according to claim 96, wherein said passenger sequence comprises a sequence selected from the group consisting of a heterologous promoter and a prokaryotic termination sequence.

20 112. The construct according to claim 96, wherein said marker sequence is selected from the group consisting of a antibiotic resistance gene or a non-antibiotic resistance gene.

25 113. The construct according to claim 112, wherein said antibiotic resistance gene is selected from the group consisting of a gentamycin resistance gene, zeocin resistance gene, kanamycin resistance gene, ampicillin resistance gene, tetracycline resistance gene, and chloramphenicol resistance gene.

30 114. The construct according to claim 112, wherein said non-antibiotic resistance gene is selected from the group consisting of an auxotrophic resistance gene, a metal ion resistance gene, a *trp* gene, and a gene encoding a green fluorescent protein.



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115. The construct according to claim 96, wherein said marker sequence comprises the kanamycin resistance gene.

116. The construct according to claim 96, wherein said construct is, or is derived  
5 from, a plasmid selected from the group consisting of pLOI2223, pLOI2224, pLOI2225, pLOI2226, pLOI2227, pLOI2228, pLOI2231, and pLOI2403.

117. A kit comprising at least one nucleic acid construct according to claim 96 and instructions for use.

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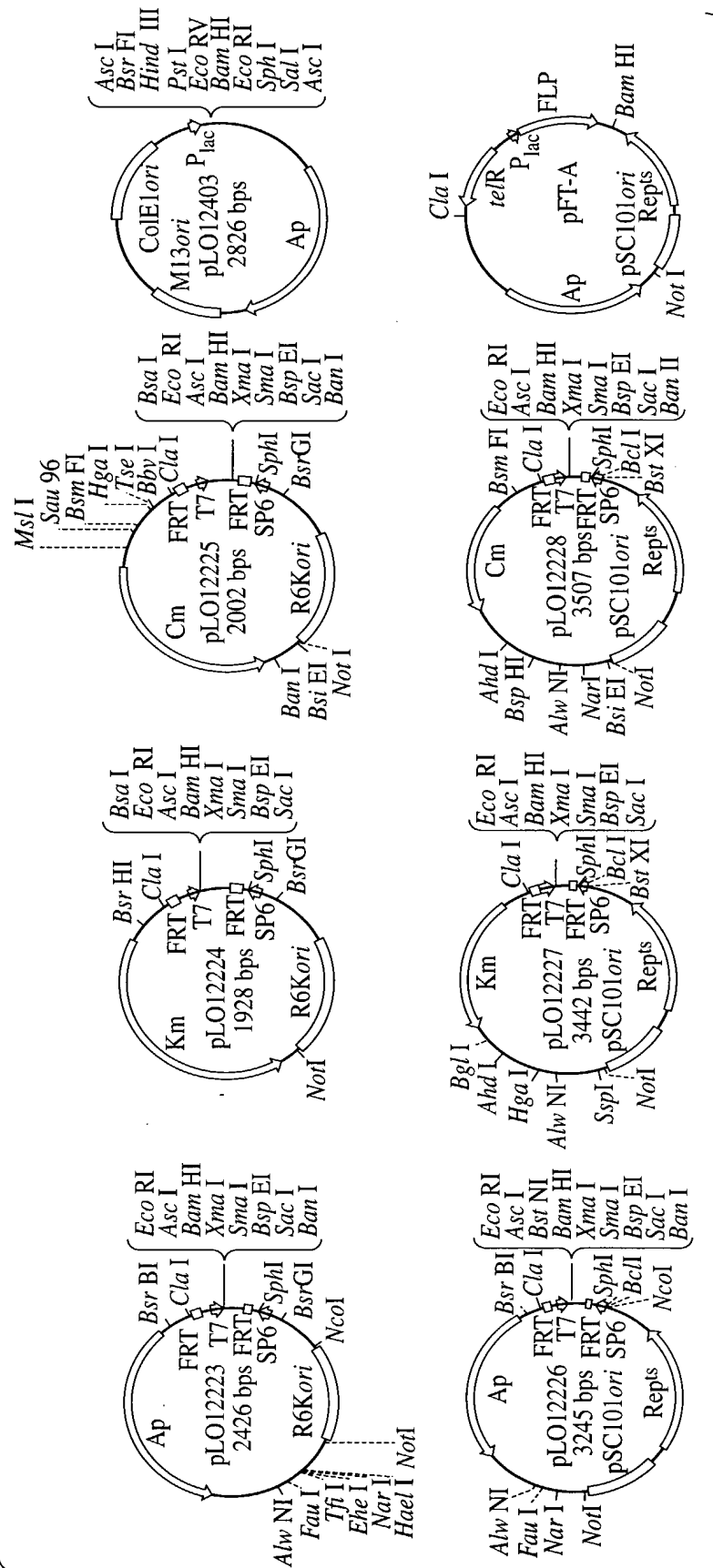


Fig. 1

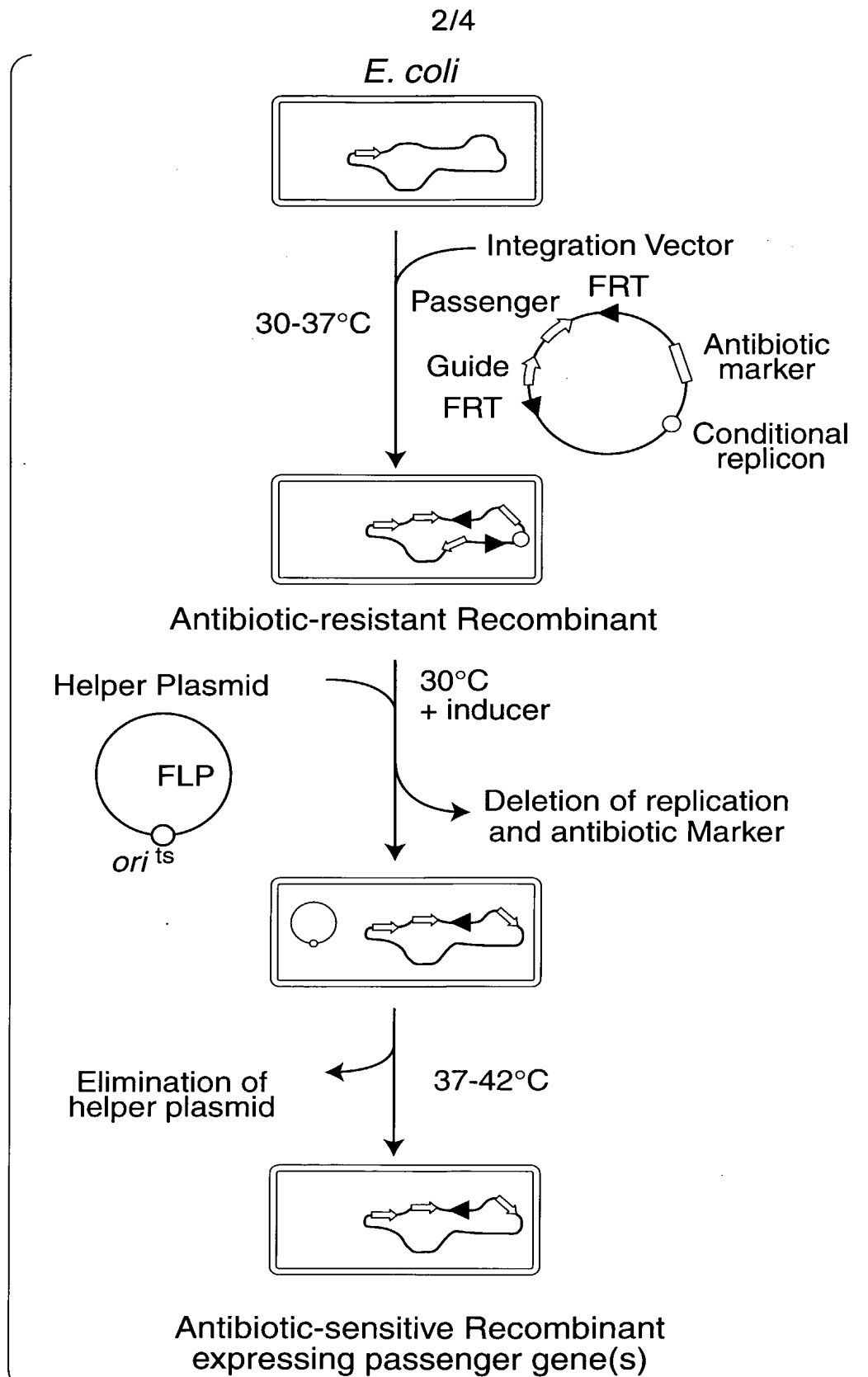


Fig. 2

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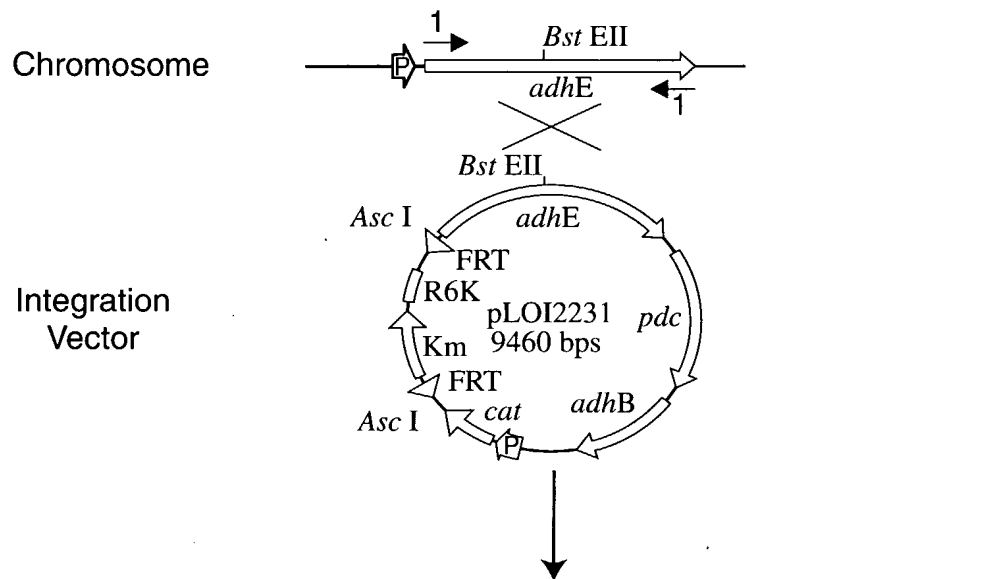


Fig. 3A

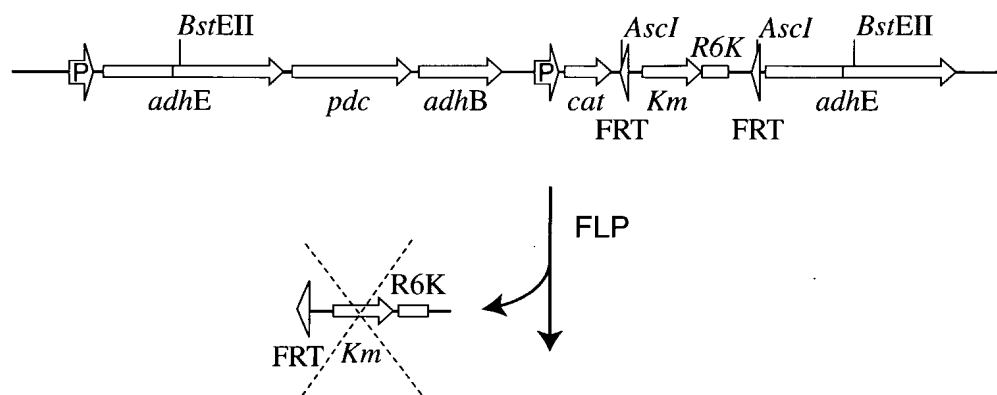


Fig. 3B

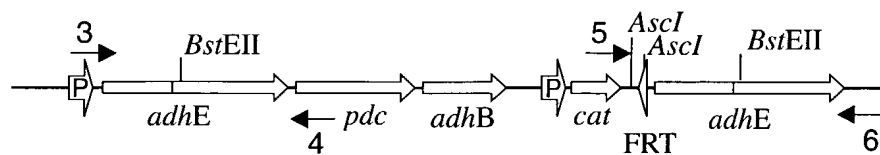


Fig. 3C

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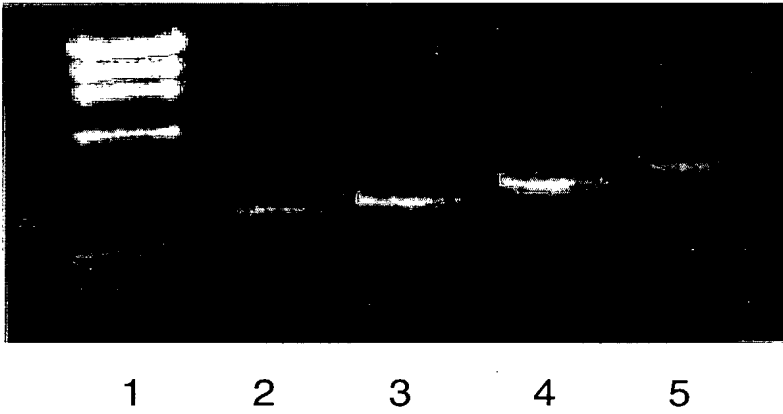


Fig. 4A

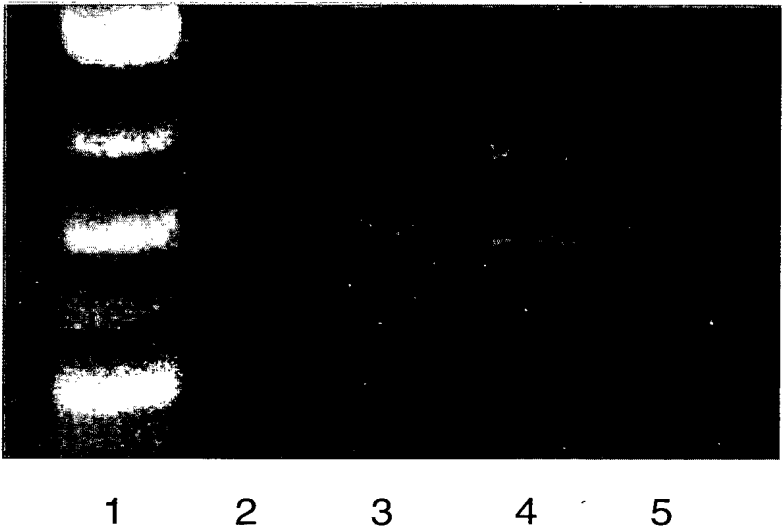


Fig. 4B

- 1 -

## SEQUENCE LISTING

&lt;110&gt; University of Florida

<120> METHODS AND COMPOSITIONS FOR THE CHROMOSOMAL  
INTEGRATION OF HETEROLOGOUS DNA

&lt;130&gt; BCI-021PC

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&lt;150&gt; 09/390,479

&lt;151&gt; 1999-09-07

&lt;160&gt; 7

&lt;170&gt; PatentIn Ver. 2.0

&lt;210&gt; 1

&lt;211&gt; 2427

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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<223> Description of Artificial Sequence: cloning  
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<223> Ampicillin-resistance gene from position 2295 to  
position 1435

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- 2 -

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780

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840

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1200

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1440

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1500

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- 3 -

1620

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1980

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2280

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2340

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&lt;211&gt; 1929

&lt;212&gt; DNA

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&lt;220&gt;

<223> Description of Artificial Sequence: cloning  
vectors

&lt;220&gt;

<223> Kanamycin-resistance gene from position 1778 to



- 4 -

position 984

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840tgagagctta gtacgttaaa catgagagct tagtacgtga aacatgagag cttagtacgt  
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1080

- 5 -

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1140

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1200

aagcagggcat cgccatgggt cagcagcaga tcctcgccgt cgggcatccg cgccttgagc  
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1320

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1380

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1440

actttctcgg caggagcaag gtgagatgac aggagatcct gcccggcac ttcgcccaat  
1500

agcagccagt cccttcccgc ttcagtgaca acgtcgagca cagctgcgca aggaacgccc  
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gtcgtggcca gccacgatag ccgcgctgcc tcgtcttggg gttcattcag ggcaccggac  
1620

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1680

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1740

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1800

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<211> 2003

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: cloning  
vectors

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- 6 -

<223> chloramphenicol-resistance gene from position 1704  
to position 1045

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120

gcttggcgcg ccaggtcgac tctagaggat ccccggggaa gatcttccgg aagatcttcc  
180

cgagctcgaa ttaattccgc gatgaattga tcccgaagt tcctattctc tagaaagtat  
240

aggaacttcg aattggtcga caagctagct tgcattgcaag cttgtattct atagtgtcac  
300

ctaaatcgta tgtgtatgat acataagggt atgtattaat tgtagccgag ttctaacgac  
360

aatatgtaca agcctaattg tgtagcatct ggcttactga agcagaccct atcatctctc  
420

tcgtaaactg ccgtcagagt cggtttggtt ggacgaacct tctgagtttc tggtaacgcc  
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720

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900

actatcaaca ggttgaactg cggatcttgc ggccgcaaaa attaaaaatg aagtttttaa  
960

tcaatctaaa gtatatatga gtaaacttgg tctgacagtt accaatgctt aatcagtgag  
1020

gcaccaataa ctgccttaaa aaaattacgc cccgccttgc cactcatcgc agtactgttg  
1080

- 7 -

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1140

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1200

ggcgaagaag ttgtccatat tggccacgtt taaatcaaaa ctggtgaaac tcaccaggg  
1260

attggctgag acgaaaaaca tattctcaat aaacccttta gggaaatagg ccaggttttc  
1320

accgtaacac gccacatctt gcgaatatat gtgtagaaac tgccggaaat cgtcgtggta  
1380

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1560

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1620

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1680

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1740

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1800

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1860

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1920

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<210> 4

<211> 3246

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: cloning  
vectors

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<223> Ampicillin-resistance gene from position 3114 to  
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gcttggcgcg ccaggtcgac tctagaggat ccccggggaa gatcttccgg aagatcttcc  
180

cgagctcgaa ttaattccgc gatgaattga tcccggaagt tcctattctc tagaaagtat  
240

aggaacttcg aattggtcga caagctagct tgcattgcaag cttggcactg gctgattcgc  
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660

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- 9 -

1020

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1440

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1800

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1860

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1920

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1980

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- 10 -

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2220

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2280

tatctcagcg atctgtctat ttcgttcacg catagttgcc tgactccccg tcgtgtagat  
2340

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2400

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3000

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3240

- 11 -

acttgc  
3246

<210> 5  
<211> 3443  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: cloning  
vectors

<220>  
<223> kanamycin-resistance gene from position 3292 to  
position 2498

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180

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720

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780



- 12 -

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1320

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1380

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- 13 -

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2580

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- 14 -

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3420

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3443

<210> 6

<211> 3508

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: cloning  
vectors

<220>

<223> chloramphenicol-resistance gene from position 3209  
to position 2550

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240

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300

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360

- 15 -

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720

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- 17 -

2640

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3508

&lt;210&gt; 7

&lt;211&gt; 9460

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: cloning  
vectors

- 18 -

&lt;220&gt;

&lt;223&gt; adhE gene from E.coli from position 1890 to position 4565

&lt;220&gt;

&lt;223&gt; pdc gene from Z.mobilis from position 4701 to position 6380

&lt;220&gt;

&lt;223&gt; adhB gene from Z.mobilis from position 6508 to position 7659

&lt;220&gt;

&lt;223&gt; chloramphenicol-resistance gene from position 8515 to position 9174

&lt;220&gt;

&lt;223&gt; kanamycin-resistance gene from position 152 to position 946

&lt;400&gt; 7

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- 19 -

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- 20 -

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- 22 -

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3960

- 23 -

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- 25 -

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- 27 -

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- 28 -

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- 29 -

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9420

tactttctag agaataggaa ctccggatca attcatcgat

9460

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/22700

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7 C12N15/85 C12N15/90 C12N1/21 //C12N15/52,C12N15/52, (C12N1/21,C12R1:19)		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 10488 A (WAHL GEOFFREY ; GORMAN STEPHEN O (US); SALK INST FOR BIOLOGICAL STU) 4 March 1999 (1999-03-04)  examples 1,3,5 figure 1  --- -/--	1,4-17, 96,99, 100,102, 106-110, 112-115, 117
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *&* document member of the same patent family		
Date of the actual completion of the international search  5 January 2001		Date of mailing of the international search report  23/01/2001
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer  Galli, I

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	--- POSFAI G. ET AL.: "Versatile insertion plasmids for targeted genome manipulations in bacteria: isolation, deletion and rescue of the pathogenicity island LEE of the Escherichia coli 0157:H7 genome." J. BACTERIOL., vol. 179, no. 13, July 1997 (1997-07), pages 4426-4428, XP002156608 figure 2	1-117
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